

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
  - TEXT CUT OFF AT TOP, BOTTOM OR SIDES
  - FADED TEXT
  - ILLEGIBLE TEXT
  - SKEWED/SLANTED IMAGES
  - COLORED PHOTOS
  - BLACK OR VERY BLACK AND WHITE DARK PHOTOS
  - GRAY SCALE DOCUMENTS
- 

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problems Mailbox.**

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
22 February 2001 (22.02.2001)

PCT

(10) International Publication Number  
**WO 01/12223 A2**

(51) International Patent Classification<sup>7</sup>: **A61K 39/39**,  
39/385, 39/35, 39/145, 39/21, A61P 31/12

(21) International Application Number: PCT/US00/22835

(22) International Filing Date: 18 August 2000 (18.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/149,768 19 August 1999 (19.08.1999) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): **DY-NAVAX TECHNOLOGIES CORPORATION** [US/US];  
Suite 100, 717 Potter Street, Berkeley, CA 94710 (US).

**Published:**

— Without international search report and to be republished upon receipt of that report.

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **VAN NEST, Gary** [US/US]; 639 Skyline Dr., Martinez, CA 94553 (US).

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(74) Agents: **POLIZZI, Catherine, M. et al.**; Morrison & Forster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

WO 01/12223 A2

(54) Title: METHODS OF MODULATING AN IMMUNE RESPONSE USING IMMUNOSTIMULATORY SEQUENCES AND COMPOSITIONS FOR USE THEREIN

(57) Abstract: The invention provides methods of modulating an immune response to a second antigen which entail administration of a first antigen and an immunostimulatory polynucleotide. Modulation of the immune response is generally manifested as stimulation of a Th1 response.

**METHODS OF MODULATING AN IMMUNE RESPONSE  
USING IMMUNOSTIMULATORY SEQUENCES AND COMPOSITIONS  
FOR USE THEREIN**

5

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of U.S. Provisional application 60/149,768, filed August 19, 1999, which is hereby incorporated herein by reference in its entirety.

10

TECHNICAL FIELD

This invention relates to the field of immunology. More specifically, it pertains to methods of modulating an immune response to one antigen by administering another antigen in conjunction with an immunostimulatory polynucleotide.

15

BACKGROUND ART

Immunization, whereby antigen is administered in order to elicit an immune response, has been successful in preventing and treating a number of major disorders, including infectious diseases and, to a more limited extent, allergies. Immunization also holds promise in other areas, such as cancer. However, in several significant contexts, there have been limitations. In some instances, administration of antigen, even when using adjuvants, fails to elicit the desired immune response. Thus, the antigen itself cannot be rendered sufficiently immunogenic. In the case of some viruses, such as flu virus, the antigen changes often, typically from season to season, necessitating reformulation of the vaccines. In other instances, the type of immune response generated by immunizing with antigen is not the desired immune response. For example, most vaccines currently in use elicit effective humoral (antibody) responses, but fail to elicit cellular responses. This has been a major hurdle in the cancer context. Finally, often it is desirable to elicit protection against a number of antigens which would be encountered simultaneously, as in the case of allergens or certain infectious diseases, or even cancer. The ability to elicit more effectively an immune response against more than one antigen could prove to significantly enhance efficacy and expand the scope of immunization.

The type of immune response generated to infection or other antigenic challenge can generally be distinguished by the subset of T helper (Th) cells involved in the response. The Th1 subset is responsible for classical cell-mediated functions such as delayed-type hypersensitivity and activation of cytotoxic T lymphocytes (CTLs), whereas the Th2 subset functions more effectively as a helper for B-cell activation. The type of immune response to an antigen is generally influenced by the cytokines produced by the cells responding to the antigen. Differences in the cytokines secreted by Th1 and Th2 cells are believed to reflect different biological functions of these two subsets.

The Th1 subset may be particularly suited to respond to viral infections, intracellular pathogens, and tumor cells because it secretes IL-2 and IFN- $\gamma$ , which activate CTLs. The Th2 subset may be more suited to respond to free-living bacteria and helminthic parasites and may mediate allergic reactions, since IL-4 and IL-5 are known to induce IgE production and eosinophil activation, respectively. In general, Th1 and Th2 cells secrete distinct patterns of cytokines and so one type of response can moderate the activity of the other type of response. A shift in the Th1/Th2 balance can result in an allergic response, for example, or, alternatively, in an increased CTL response.

For many infectious diseases, such as tuberculosis and malaria, Th2-type responses are of little protective value against infection. Proposed vaccines using small peptides derived from the target antigen and other currently used antigenic agents that avoid use of potentially infective intact viral particles, do not always elicit the immune response necessary to achieve a therapeutic effect. The lack of a therapeutically effective human immunodeficiency virus (HIV) vaccine is an unfortunate example of this failure. Protein-based vaccines typically induce Th2-type immune responses, characterized by high titers of neutralizing antibodies but without significant cell-mediated immunity.

Moreover, some types of antibody responses are inappropriate in certain indications, most notably in allergy where an IgE antibody response can result in anaphylactic shock. Generally, allergic responses also involve Th2-type immune responses. Allergic responses, including those of allergic asthma, are characterized by an early phase response, which occurs within seconds to minutes of allergen exposure and is characterized by cellular degranulation, and a late phase response, which occurs 4 to 24 hours later and is characterized by infiltration of eosinophils into the site of allergen exposure. Specifically, during the early phase of the allergic response, allergen cross-links

IgE antibodies on basophils and mast cells, which in turn triggers degranulation and the subsequent release of histamine and other mediators of inflammation from mast cells and basophils. During the late phase response, eosinophils infiltrate into the site of allergen exposure (where tissue damage and dysfunction result).

5           Antigen immunotherapy for allergic disorders involves the subcutaneous injection of small, but gradually increasing amounts, of antigen. Such immunization treatments present the risk of inducing IgE-mediated anaphylaxis and do not efficiently address the cytokine-mediated events of the allergic late phase response. Thus far, this approach has yielded only limited success.

10           Administration of certain DNA sequences, generally known as immunostimulatory sequences or "ISS," induces an immune response with a Th1-type bias as indicated by secretion of Th1-associated cytokines. Administration of an immunostimulatory polynucleotide with an antigen results in a Th1-type immune response to the administered antigen. Roman et al. (1997) *Nature Med.* 3:849-854. For example, mice injected  
15 intradermally with *Escherichia coli* (*E. coli*)  $\beta$ -galactosidase ( $\beta$ -Gal) in saline or in the adjuvant alum responded by producing specific IgG1 and IgE antibodies, and CD4<sup>+</sup> cells that secreted IL-4 and IL-5, but not IFN- $\gamma$ , demonstrating that the T cells were predominantly of the Th2 subset. However, mice injected intradermally (or with a tyne skin scratch applicator) with plasmid DNA (in saline) encoding  $\beta$ -Gal and containing an  
20 ISS responded by producing IgG2a antibodies and CD4<sup>+</sup> cells that secreted IFN- $\gamma$ , but not IL-4 and IL-5, demonstrating that the T cells were predominantly of the Th1 subset. Moreover, specific IgE production by the plasmid DNA-injected mice was reduced 66-75%. Raz et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:5141-5145. In general, the response to naked DNA immunization is characterized by production of IL-2, TNF $\alpha$  and IFN- $\gamma$  by  
25 antigen-stimulated CD4<sup>+</sup> T cells, which is indicative of a Th1-type response. This is particularly important in treatment of allergy and asthma as shown by the decreased IgE production. The ability of immunostimulatory polynucleotides to stimulate a Th1-type immune response has been demonstrated with bacterial antigens, viral antigens and with allergens (see, for example, WO 98/55495).

30           Other references describing ISS include: Krieg et al. (1989) *J. Immunol.* 143:2448-2451; Tokunaga et al. (1992) *Microbiol. Immunol.* 36:55-66; Kataoka et al. (1992) *Jpn. J. Cancer Res.* 83:244-247; Yamamoto et al. (1992) *J. Immunol.* 148:4072-4076; Mojcić et

- al. (1993) *Clin. Immuno. and Immunopathol.* 67:130-136; Branda et al. (1993) *Biochem. Pharmacol.* 45:2037-2043; Pisetsky et al. (1994) *Life Sci.* 54(2):101-107; Yamamoto et al. (1994a) *Antisense Research and Development.* 4:119-122; Yamamoto et al. (1994b) *Jpn. J. Cancer Res.* 85:775-779; Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523;
- 5 Kimura et al. (1994) *J. Biochem. (Tokyo)* 116:991-994; Krieg et al. (1995) *Nature* 374:546-549; Pisetsky et al. (1995) *Ann. N.Y. Acad. Sci.* 772:152-163; Pisetsky (1996a) *J. Immunol.* 156:421-423; Pisetsky (1996b) *Immunity* 5:303-310; Zhao et al. (1996) *Biochem. Pharmacol.* 51:173-182; Yi et al. (1996) *J. Immunol.* 156:558-564; Krieg (1996) *Trends Microbiol.* 4(2):73-76; Krieg et al. (1996) *Antisense Nucleic Acid Drug Dev.* 6:133-139;
- 10 Klinman et al. (1996) *Proc. Natl. Acad. Sci. USA.* 93:2879-2883; Raz et al. (1996); Sato et al. (1996) *Science* 273:352-354; Stacey et al. (1996) *J. Immunol.* 157:2116-2122; Ballas et al. (1996) *J. Immunol.* 157:1840-1845; Branda et al. (1996) *J. Lab. Clin. Med.* 128:329-338; Sonehara et al. (1996) *J. Interferon and Cytokine Res.* 16:799-803; Klinman et al. (1997) *J. Immunol.* 158:3635-3639; Sparwasser et al. (1997) *Eur. J. Immunol.* 27:1671-
- 15 1679; Roman et al. (1997); Carson et al. (1997) *J. Exp. Med.* 186:1621-1622; Chace et al. (1997) *Clin. Immunol. and Immunopathol.* 84:185-193; Chu et al. (1997) *J. Exp. Med.* 186:1623-1631; Lipford et al. (1997a) *Eur. J. Immunol.* 27:2340-2344; Lipford et al. (1997b) *Eur. J. Immunol.* 27:3420-3426; Weiner et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:10833-10837; Macfarlane et al. (1997) *Immunology* 91:586-593; Schwartz et al. (1997)
- 20 *J. Clin. Invest.* 100:68-73; Stein et al. (1997) *Antisense Technology*, Ch. 11 pp. 241-264, C. Lichtenstein and W. Nellen, Eds., IRL Press; Wooldridge et al. (1997) *Blood* 89:2994-2998; Leclerc et al. (1997) *Cell. Immunol.* 179:97-106; Kline et al. (1997) *J. Invest. Med.* 45(3):282A; Yi et al. (1998a) *J. Immunol.* 160:1240-1245; Yi et al. (1998b) *J. Immunol.* 160:4755-4761; Yi et al. (1998c) *J. Immunol.* 160:5898-5906; Yi et al. (1998d) *J. Immunol.*
- 25 161:4493-4497; Krieg (1998) *Applied Antisense Oligonucleotide Technology* Ch. 24, pp. 431-448, C.A. Stein and A.M. Krieg, Eds., Wiley-Liss, Inc.; Krieg et al. (1998a) *Trends Microbiol.* 6:23-27; Krieg et al. (1998b) *J. Immunol.* 161:2428-2434; Krieg et al. (1998c) *Proc. Natl. Acad. Sci. USA* 95:12631-12636; Spiegelberg et al. (1998) *Allergy* 53(45S):93-97; Horner et al. (1998) *Cell Immunol.* 190:77-82; Jakob et al. (1998) *J. Immunol.*
- 30 161:3042-3049; Redford et al. (1998) *J. Immunol.* 161:3930-3935; Weeratna et al. (1998) *Antisense & Nucleic Acid Drug Development* 8:351-356; McCluskie et al. (1998) *J. Immunol.* 161(9):4463-4466; Gramzinski et al. (1998) *Mol. Med.* 4:109-118; Liu et al.

(1998) *Blood* 92:3730-3736; Moldoveanu et al. (1998) *Vaccine* 16: 1216-1224; Brazolot Milan et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:15553-15558; Briode et al. (1998) *J. Immunol.* 161:7054-7062; Briode et al. (1999) *Int. Arch. Allergy Immunol.* 118:453-456; Kovarik et al. (1999) *J. Immunol.* 162:1611-1617; Spiegelberg et al. (1999) *Pediatr. Pulmonol. Suppl.* 18:118-121; Martin-Orozco et al. (1999) *Int. Immunol.* 11:1111-1118; EP 468,520; WO 96/02555; WO 97/28259; WO 98/16247; WO 98/18810; WO 98/37919; WO 98/40100; WO 98/52581; WO 98/55495; WO 98/55609 and WO 99/11275. See also Elkins et al. (1999) *J. Immunol.* 162:2291-2298; WO 98/52962; WO 99/33488; WO 99/33868; WO 99/51259 and WO 99/62923. See also Zimmermann et al. (1998) *J. Immunol.* 160:3627-3630; Krieg (1999) *Trends Microbiol.* 7:64-65 and U.S. Patent Nos. 5,663,153, 5,723,335 and 5,849,719.

As pathogens and allergy-inducing sources generally contain more than one immunogen or allergen, respectively, it would be especially desirable to enhance and/or modulate an immune response to the multiple antigens that are encountered upon, for example, viral infection or exposure to an allergy-inducing source. The present invention provides methods that can be employed in these contexts.

All publications and references cited herein are hereby incorporated by reference in their entirety.

20

#### DISCLOSURE OF THE INVENTION

The present invention provides methods which achieve modulation of an immune response against a second antigen, which is mediated by administration of a first antigen in conjunction with an immunostimulatory polynucleotide sequence.

Accordingly, in one aspect, the invention provides methods of modulating an immune response to a second antigen in an individual, comprising administering to the individual (preferably a mammal, more preferably a human) an immunomodulatory polynucleotide comprising an immunostimulatory sequence (ISS) and a first antigen, wherein the ISS-containing immunomodulatory polynucleotide and first antigen are administered in an amount sufficient to modulate an immune response to the second antigen upon exposure to the second antigen. In embodiments in which second antigen is not administered (i.e., first antigen is administered in absence of administration of second

antigen), exposure to the second antigen is concurrent with first antigen. Preferably, the modulation of the immune response is stimulation of a Th1 response to the second antigen.

The first antigen and ISS containing polynucleotide may be co-administered in a variety of forms, including spatially proximate to one another (i.e., a generally fixed spatial relationship) or in an admixture. An antigen and ISS-containing polynucleotide may be proximately associated by conjugation, encapsulation, adsorption onto a surface, or linkage to a platform molecule. The first antigen can be any of a number of molecules, including moieties derived from infectious agents, such as virus or bacteria, allergens and carrier molecules. The first antigen may be associated with a carrier molecule.

In some aspects, the first antigen and ISS-containing polynucleotide are proximately associated, and are administered with a second antigen (i.e., one or more additional antigens). The second antigen may be any of a number of moieties, as described herein. Such administration results in modulation of an immune response to the second antigen(s), preferably a Th1 response.

The first antigen and ISS-containing polynucleotide (with or without second antigen) may be administered at any of a variety of times before and/or during exposure to the second antigen. In some embodiments, the ISS-containing polynucleotide and first antigen are administered upon exposure to the second antigen. The second antigen may or may not be encountered at the same site as the site of administration of the first antigen and ISS-containing polypeptide.

ISS are described herein, and generally comprise the sequence 5'-cytosine, guanine-3', more particularly comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3' (such as 5'-AACGTT-3'). In some embodiments, the ISS generally comprises the sequence 5'-T,C,G-3'.

The invention also provides immunogenic compositions comprising a carrier molecule and an immunomodulatory polynucleotide comprising an ISS, preferably further comprising a pharmaceutically acceptable excipient. These compositions may further include an antigen (other than the carrier molecule). The invention also provides immunogenic compositions comprising an ISS-containing polynucleotide proximately associated with a first antigen and further comprising one or more additional antigens.



### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a bar graph depicting the effect of administration of an ISS-Amb a 1 conjugate ("AIC") on  $\beta$ -gal IgG2a response (which indicates a Th1 response) in mice. Left hand bar, administration of  $\beta$ -gal alone; middle bar, administration of  $\beta$ -gal with 1  $\mu$ g conjugate; right hand bar, administration of  $\beta$ -gal and 10  $\mu$ g conjugate.

### MODES FOR CARRYING OUT THE INVENTION

We have discovered that administration of a first antigen with an immunomodulatory polynucleotide comprising an immunostimulatory sequence(s) (ISS) elicits an immune response, particularly a Th1 response, to a second antigen. Modulating the immune response to an additional antigen in response to administration of a first antigen offers distinct benefits and advantages. This immunotherapeutic approach obviates or at least reduces the need for having to design and manufacture various formulations reflecting different antigenic compositions. It also mitigates the requirement for identification of all relevant antigens for immunotherapy. For example, allergy desensitization therapy could be accomplished by administration of an ISS-containing polynucleotide and just one antigen. This is especially significant in some contexts, such as with cockroach, which contains many antigens. This may also be beneficial for relief from different allergens which, due to seasonal and geographical parameters, are encountered together. Further, with respect to immunization against pathogens (whether prophylactic or therapeutic) rapid mutations in antigenic proteins, such as coat proteins, would not necessitate identification of the changes and concomitant reformulation of vaccines to reflect the mutations. In the context of antigens administered in the form of antigen-carrier conjugates, such as oligosaccharide antigens, administration of one such conjugate with an ISS-containing polynucleotide would modulate the immune response to another antigen when administered with the same protein carrier. The immune response to the second antigen could be obtained without the need to generate additional formulations.

We have further discovered that one or more benefits associated with administration of an ISS-containing polynucleotide spatially proximate to a first antigen, namely immune modulation at a significantly lower dose, stronger interferon  $\gamma$  response and an enhanced CTL response, are also observed with respect to a second antigen upon administration of a second antigen (i.e., one or more additional antigens) with an ISS-containing

polynucleotide spatially proximate to a first antigen. This finding has significant, positive implications in terms of dosing (in that lower dosages may be required to obtain the desired response) as well as practical, manufacturing considerations (in that there is a reduced need to link additional antigens to an ISS-containing polynucleotide in order to obtain the benefit of such linkage).

#### General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Weir & C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *The Immunoassay Handbook* (David Wild, ed., Stockton Press NY, 1994); and *Methods of Immunological Analysis* (R. Masseyeff, W.H. Albert, and N.A. Staines, eds., Weinheim: VCH Verlags gesellschaft mbH, 1993).

#### Definitions

As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise. For example, "a" second antigen includes one or more additional antigens.

The term "ISS" as used herein refers to polynucleotide sequences that effect a measurable immune response as measured *in vitro*, *in vivo* and/or *ex vivo*. Examples of measurable immune responses include, but are not limited to, antigen-specific antibody production, secretion of cytokines, activation or expansion of lymphocyte populations such as NK cells, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, B lymphocytes, and the like.

Preferably, the ISS sequences preferentially activate a Th1-type response. A polynucleotide for use in methods of the invention contains at least one ISS.

As used interchangeably herein, the terms "polynucleotide" and "oligonucleotide" include single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), modified oligonucleotides and oligonucleosides or combinations thereof. The oligonucleotide can be linearly or circularly configured, or the oligonucleotide can contain both linear and circular segments.

The term "immunomodulatory" or "modulating an immune response" as used herein includes immunostimulatory as well as immunosuppressive effects.

Immunostimulatory effects include, but are not limited to, those that directly or indirectly enhance cellular or humoral immune responses. Examples of immunostimulatory effects include, but are not limited to, increased antigen-specific antibody production; activation or proliferation of a lymphocyte population such as NK cells, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, macrophages and the like; increased synthesis of immunostimulatory cytokines including, but not limited to, IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , TNF- $\alpha$  and the like. Immunosuppressive effects include those that directly or indirectly decrease cellular or humoral immune responses. Examples of immunosuppressive effects include, but are not limited to, a reduction in antigen-specific antibody production such as reduced IgE production; activation of lymphocyte or other cell populations that have immunosuppressive activities such as those that result in immune tolerance; and increased synthesis of cytokines that have suppressive effects toward certain cellular functions. One example of this is IFN- $\gamma$ , which appears to block IL-4 induced class switch to IgE and IgG1, thereby reducing the levels of these antibody subclasses.

The term "conjugate" refers to a complex in which an ISS-containing polynucleotide and an antigen are linked. Such conjugate linkages include covalent and/or non-covalent linkages.

The term "antigen" means a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins, glycoproteins, polysaccharides, gangliosides and lipids; portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic. Haptens are included within the scope of "antigen." A hapten is a low molecular weight compound that

is not immunogenic by itself but is rendered immunogenic when conjugated with an immunogenic molecule containing antigenic determinants.

5 A "second antigen" refers to an antigen other than a first antigen (including a different antigenic region within the same polypeptide) which is encountered (i.e., by an environmental exposure) by and/or administered to an individual, and against which an immune response is modulated by the methods of the invention. As described herein, in some embodiments, a second antigen is administered in addition to an ISS-containing polynucleotide proximately associated with a first antigen.

10 A "carrier molecule" refers to an immunogenic molecule used in association with an antigen, usually by covalent linkage, to facilitate, cause and/or modulate an immune response to the antigen. Examples of carriers are provided herein.

"Adjuvant" refers to a substance which, when added to an immunogenic agent such as antigen, nonspecifically enhances or potentiates an immune response to the agent in the recipient host upon exposure to the mixture.

15 The term "peptide" are polypeptides that are of sufficient length and composition to effect a biological response, e.g. antibody production or cytokine activity whether or not the peptide is a hapten. Typically, the peptides are of at least six amino acid residues in length. The term "peptide" further includes modified amino acids, such modifications including, but not limited to, phosphorylation, glycosylation, pegylation, lipidization and methylation.

20 A "conserved" or "constant" polypeptide is a term understood by those in the art and generally refers to a polypeptide (or a region or domain of a polypeptide) that does not mutate, or change its sequence, at an appreciable rate. A polypeptide, even a polypeptide that has variable regions, may comprise one or more conserved regions, or domains, and the terms "conserved" or "constant" or "preserved" polypeptides encompasses polypeptides consisting of, as well as comprising, these conserved regions. Thus a "conserved" or "constant" polypeptide may be a complete or partial sequence. The term "constant domain" includes regions of antigens that are not prone to vary between strains and/or species of virus. Generally, an amino acid sequence comparison indicates conserved polypeptides (including conserved regions of polypeptides), although three-dimensional conformation may be conserved although sequences appear to be different. For purposes of this invention, "conserved" usually pertains to sequence conservation. As understood in

the art, a "constant" polypeptide is distinguished from a "variable" polypeptide (which includes variable regions or domains), which refers to a polypeptide that does mutate at an appreciable rate. Thus the terms "constant" and "variable" are relative. Examples of a variable peptides are some viral coat proteins. Coat proteins include, but are not limited to, HIV envelope proteins, influenza virus hemagglutinin (HA) protein and influenza virus neuraminidase (NA) protein. As this definition makes clear, a conserved viral core protein may be the entire core sequence or a portion of that sequence.

The term "immunomodulatory facilitator" refers to molecules which support and/or enhance the immunomodulatory activity of an ISS. Examples of immunomodulatory facilitators include co-stimulatory molecules, such as cytokines, and/or adjuvants.

A "delivery molecule" or "delivery vehicle" is a chemical moiety which facilitates, permits, and/or enhances delivery of an ISS and/or antigen to a particular site and/or with respect to particular timing. A delivery vehicle may or may not additionally stimulate an immune response.

An "allergic response to antigen" means an immune response generally characterized by the generation of eosinophils and/or antigen-specific IgE and their resultant effects. As is well-known in the art, IgE binds to IgE receptors on mast cells and basophils. Upon later exposure to the antigen recognized by the IgE, the antigen cross-links the IgE on the mast cells and basophils causing degranulation of these cells. It is understood and intended that the terms "allergic response to antigen", "allergy", and "allergic condition" are equally appropriate for application of some of the methods of the invention. Further, it is understood and intended that the methods of the invention include those that are equally appropriate for prevention of an allergic response as well as treating a pre-existing allergic condition.

As used herein, the term "allergen" means an antigen or antigenic portion of a molecule, usually a protein, which elicits an allergic response upon exposure to a subject. Typically the subject is allergic to the allergen as indicated, for instance, by the wheal and flare test or any method known in the art. A molecule is said to be an allergen even if only a small subset of subjects exhibit an allergic (e.g., IgE) immune response upon exposure to the molecule. A number of isolated allergens are known in the art. These include, but are not limited to, those provided in Table 1 herein.

The term "desensitization" refers to the process of the administration of increasing doses of an allergen to which the subject has demonstrated sensitivity. Examples of allergen doses used for desensitization are known in the art, see, for example, Fornadley (1998) *Otolaryngol. Clin. North Am.* 31:111-127.

5        An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, humans, farm animals, sport animals, rodents and pets.

10        An "effective amount" or a "sufficient amount" of a substance is that amount sufficient to effect beneficial or desired results, including clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. In the context of administering a composition that modulates an immune response to a second antigen, an effective amount of a composition comprising an ISS and a first antigen is an amount sufficient to achieve such a modulation as compared to the immune response obtained when the second antigen is administered alone. An effective amount can be administered  
15 in one or more administrations.

The term "co-administration" as used herein refers to the administration of at least two different substances sufficiently close in time to modulate an immune response. Preferably, co-administration refers to simultaneous administration of at least two different substances.

20        "Stimulation" of an immune response, such as Th1 response, means an increase in the response, which can arise from eliciting and/or enhancement of a response.

A second antigen which is encountered by an individual "concurrently" with a first antigen generally means at approximately the same time, and does not necessarily mean (although the term does encompass) exactly the same time. For example, a second antigen  
25 may be encountered within a number of hours, days, or weeks of encountering the first antigen. The second antigen may or may not be encountered at the same site (or location) that the first antigen is encountered. Thus, the second antigen may be encountered at the same site that the first antigen is encountered within a number of hours, days, or weeks of encountering the first antigen. Alternatively, the second antigen may be encountered at a  
30 different site than the site that the first antigen is encountered within a number of hours, days, or weeks of encountering the first antigen.

An ISS-containing polynucleotide and an antigen that are "proximately associated", in "proximate association" or "spatially proximate" refers to an arrangement which maintains the ISS-containing polynucleotide and antigen at an average distance. Generally, and most preferably, this distance is effective to enhance an immune response generated compared to the administration of the ISS-containing polynucleotide and antigen as an admixture. As described herein, there are various ways to effect "proximate association", such as conjugation, encapsulation, adsorption, and via a platform molecule.

### *Methods of the invention*

The invention provides methods of modulating an immune response to a second antigen in an individual, preferably a mammal, more preferably a human, comprising administering to the individual an immunomodulatory polynucleotide comprising an ISS and a first antigen. For purposes of this invention, the immunomodulatory (i.e., ISS-containing) polynucleotide and first antigen are administered in an amount sufficient to modulate an immune response to a second antigen upon exposure to that antigen.

For purposes of this invention, antigens are introduced to an individual in either or both of two phases: administration, in which a first antigen and ISS-containing polynucleotide (with or without a second antigen) are deliberately introduced; and encounter, when one or more antigens are introduced to the individual via some form of environmental exposure. For purposes of this invention, an individual may or may not have been previously exposed to the first or second antigen before administration of the ISS-containing polynucleotide and first antigen (i.e., the individual may or may not be naïve with respect to the first and/or second antigens).

In some embodiments, a second antigen is not administered with the first antigen and ISS-containing polynucleotide. In these embodiments, the second antigen is encountered by the individual in the presence of (i.e., concomitantly or concurrently with) the first antigen. That is, for the methods of this invention to be effective, after administration of the ISS-containing polynucleotide and first antigen, the individual should concurrently encounter the first and second antigen in order to elicit the desired immune response to second antigen.

In other embodiments, a second antigen is administered with an ISS-containing polynucleotide which is proximately associated with a first antigen. In these embodiments,

the second antigen need not be encountered by the individual in the presence of (i.e., concomitantly or concurrently with) the first antigen (i.e., the second antigen(s) may be encountered alone). Methods to accomplish proximate association of antigen and ISS-containing polynucleotide are discussed below.

5 Descriptions of ISS and first antigens which may be used in the present invention are provided below. The ISS and the first antigen can be co-administered in an admixture sufficiently close in time so as to modulate an immune response to the antigen (i.e., co-administered). Preferably, the ISS and the first antigen are administered simultaneously. In some embodiments, the polynucleotide comprising an ISS is linked to, or proximately  
10 associated to, a first antigen. Also described below are various ways of proximately associating the immunomodulatory polynucleotide with first antigen.

It is understood that an immune response may be elicited against one or more additional antigens. Thus, the invention encompasses methods by which an immune response is elicited against a third, fourth, fifth, etc., antigen. Preferably, the immune  
15 response is modulated by stimulation of a Th1 response. In other embodiments, the immune response is modulated by suppression of the Th2 response. An immune response may be a primary response and/or a memory T cell response.

In some embodiments, administration of the first antigen and ISS-containing polynucleotide occurs at the same site as the second antigen is and/or will be encountered.  
20 For example, if the second antigen is encountered at the mucosa, such as lung or vaginal tissue, then the immunomodulatory polynucleotide and first antigen are administered to the relevant mucosa. In other embodiments, the immunomodulatory polynucleotide and first antigen are administered at a site other than where the second antigen is encountered. For example, in the case of certain allergens, the immunomodulatory polynucleotide-first  
25 antigen is administered by injection, while the second antigen is encountered through the nasal passages.

### ISS

In accordance with the present invention, the immunomodulatory polynucleotide  
30 contains at least one ISS, and can contain multiple ISSs. The ISSs can be adjacent within the polynucleotide, or they can be separated by additional nucleotide bases within the polynucleotide.



ISS have been described in the art and may be readily identified using standard assays which indicate various aspects of the immune response, such as cytokine secretion, antibody production, NK cell activation and T cell proliferation. See, e.g., WO 97/28259; WO 98/16247; WO 99/11275; Krieg et al. (1995); Yamamoto et al. (1992); Ballas et al. (1996); Klinman et al. (1997); Sato et al. (1996); Pisetsky (1996a); Shimada et al. (1986) *Jpn. J. Cancer Res.* 77:808-816; Cowdery et al. (1996) *J. Immunol.* 156:4570-4575; Roman et al. (1997); and Lipford et al. (1997a).

The ISS can be of any length greater than 6 bases or base pairs and generally comprises the sequence 5'-cytosine, guanine-3', more particularly comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3' (such as 5'-AACGTT-3'), preferably greater than 15 bases or base pairs, more preferably greater than 20 bases or base pairs in length. An ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. An ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'. As is evident from the following embodiments, an ISS may also comprise 5'-T,C,G-3'.

In some embodiments, the ISS comprises any of the following sequences:  
 GACGCTCC; GACGTCCC; GACGTTCC; GACGCCCC; AGCGTTCC; AGCGCTCC;  
 AGCGTCCC; AGCGCCCC; AACGTCCC; AACGCCCC; AACGTTCC; AACGCTCC;  
 GCGGTTCC; GCGGCTCC; GCGGTCCC; GCGGCCCC; GACGCTCG; GACGTCCG;  
 GACGCCCCG; GACGTTCG; AGCGCTCG; AGCGTTCG; AGCGTCCG; AGCGCCCCG;  
 AACGTCCG; AACGCCCCG; AACGTTCG; AACGCTCG; GCGGTTCCG; GCGGCTCG;  
 GCGGTCCG; GCGGCCCCG. In some embodiments, the immunomodulatory polynucleotide comprises the sequence 5'-TGACTGTGAACGTTTCGAGATGA-3' (SEQ ID NO:1). In other embodiments, the ISS comprises any of the sequences:  
 5'-TGACCGTGAACGTTTCGAGATGA-3' (SEQ ID NO:2);  
 5'-TCATCTCGAACGTTCCACAGTCA-3' (SEQ ID NO:3);  
 5'-TGACTGTGAACGTTCCAGATGA-3' (SEQ ID NO:4);  
 5'-TCCATAACGTTTCGCTAACGTTTCGTC-3' (SEQ ID NO:5);  
 5'-TGACTGTGAABGTTCCAGATGA-3' (SEQ ID NO:6), where B is 5-bromocytosine ;  
 5'-TGACTGTGAABGTTTCGAGATGA-3' (SEQ ID NO:7), where B is 5-bromocytosine  
 and 5'-TGACTGTGAABGTTTCGAGATGA-3' (SEQ ID NO:8), where B is 5-bromocytosine.

An ISS and/or ISS-containing polynucleotide may contain modifications. Modifications of ISS include any known in the art, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group. Various such modifications are described below.

An ISS may be single stranded or double stranded DNA, as well as single or double-stranded RNA or other modified polynucleotides. An ISS may or may not include one or more palindromic regions, which may be present in the hexameric motif described above or may extend beyond the motif. An ISS may comprise additional flanking sequences, some of which are described herein. An ISS may contain naturally-occurring or modified, non-naturally occurring bases, and may contain modified sugar, phosphate, and/or termini. For example, phosphate modifications include, but are not limited to, methyl phosphonate, phosphorothioate, phosphoramidate (bridging or non-bridging), phosphotriester and phosphorodithioate and may be used in any combination. Other non-phosphate linkages may also be used. Preferably, oligonucleotides of the present invention comprise phosphorothioate backbones. Sugar modifications known in the field, such as 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras and others described herein, may also be made and combined with any phosphate modification. Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the ISS (e.g., 5-bromocytosine, 5-chlorocytosine, 5-fluorocytosine, 5-iodocytosine).

The ISS can be synthesized using techniques and nucleic acid synthesis equipment which are well known in the art including, but not limited to, enzymatic methods, chemical methods, and the degradation of larger oligonucleotide sequences. See, for example, Ausubel et al. (1987); and Sambrook et al. (1989). When assembled enzymatically, the individual units can be ligated, for example, with a ligase such as T4 DNA or RNA ligase. U.S. Patent No. 5,124,246. Oligonucleotide degradation can be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675.

The ISS can also be isolated using conventional polynucleotide isolation procedures. Such procedures include, but are not limited to, hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences, antibody screening of

expression libraries to detect shared structural features and synthesis of particular native sequences by the polymerase chain reaction.

5 Circular ISS can be isolated, synthesized through recombinant methods, or chemically synthesized. Where the circular ISS is obtained through isolation or through recombinant methods, the ISS will preferably be a plasmid. The chemical synthesis of smaller circular oligonucleotides can be performed using any method described in the literature. See, for instance, Gao et al. (1995) *Nucleic Acids Res.* 23:2025-2029; and Wang et al. (1994) *Nucleic Acids Res.* 22:2326-2333.

10 The techniques for making oligonucleotides and modified oligonucleotides are known in the art. Naturally occurring DNA or RNA, containing phosphodiester linkages, is generally synthesized by sequentially coupling the appropriate nucleoside phosphoramidite to the 5'-hydroxy group of the growing oligonucleotide attached to a solid support at the 3'-end, followed by oxidation of the intermediate phosphite triester to a phosphate triester. Once the desired oligonucleotide sequence has been synthesized, the oligonucleotide is removed from the support, the phosphate triester groups are deprotected to phosphate diesters and the nucleoside bases are deprotected using aqueous ammonia or other bases. See, for example, Beaucage (1993) "Oligodeoxyribonucleotide Synthesis" in in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) Humana Press, Totowa, NJ; Warner et al. (1984) *DNA* 3:401 and U.S. Patent No. 4,458,066.

20 The ISS can also contain phosphate-modified oligonucleotides. Synthesis of polynucleotides containing modified phosphate linkages or non-phosphate linkages is also known in the art. For a review, see Matteucci (1997) "Oligonucleotide Analogs: an Overview" in *Oligonucleotides as Therapeutic Agents*, (D.J. Chadwick and G. Cardew, ed.) John Wiley and Sons, New York, NY. The phosphorous derivative (or modified phosphate group) which can be attached to the sugar or sugar analog moiety in the oligonucleotides of the present invention can be a monophosphate, diphosphate, triphosphate, alkylphosphonate, phosphorothioate, phosphorodithioate or the like. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified nucleotides and oligonucleotides, *per se*, is also known and need not be described here in detail. Peyrottes et al. (1996) *Nucleic Acids Res.* 24:1841-1848; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24:2318-2323; and Schultz et al. (1996) *Nucleic Acids Res.* 24:2966-2973. For example, synthesis of phosphorothioate oligonucleotides is similar to that

described above for naturally occurring oligonucleotides except that the oxidation step is replaced by a sulfurization step (Zon (1993) "Oligonucleoside Phosphorothioates" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) Humana Press, pp. 165-190). Similarly the synthesis of other phosphate analogs, such as phosphotriester (Miller et al. (1971) *JACS* 93:6657-6665), non-bridging phosphoramidates (Jager et al. (1988) *Biochem.* 27:7247-7246), N3' to P5' phosphoramidates (Nelson et al. (1997) *JOC* 62:7278-7287) and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified oligonucleotides can also be used (Stirchak et al. (1989) *Nucleic Acids Res.* 17:6129-6141). Oligonucleotides with phosphorothioate backbones can be more immunogenic than those with phosphodiester backbones and appear to be more resistant to degradation after injection into the host. Braun et al. (1988) *J. Immunol.* 141:2084-2089; and Latimer et al. (1995) *Mol. Immunol.* 32:1057-1064.

An ISS can comprise ribonucleotides (containing ribose as the only or principal sugar component), deoxyribonucleotides (containing deoxyribose as the principal sugar component), or, as is known in the art, modified sugars or sugar analogs can be incorporated in the ISS. Thus, in addition to ribose and deoxyribose, the sugar moiety can be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar can be in pyranosyl or in a furanosyl form. In the ISS, the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-O-alkylribose, and the sugar can be attached to the respective heterocyclic bases either in  $\alpha$  or  $\beta$  anomeric configuration. Sugar modifications include, but are not limited to, 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) *per se* is known, and need not be described here, except to the extent such preparation can pertain to any specific example. Sugar modifications may also be made and combined with any phosphate modification in the preparation of an ISS.

The heterocyclic bases, or nucleic acid bases, which are incorporated in the ISS can be the naturally-occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally-occurring and synthetic modifications of said principal bases.

Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and that as long as other criteria of the present invention are satisfied, the ISS can include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. Preferably, however, the heterocyclic base in the ISS includes, but is not limited to, uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2,3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the ISS via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

The ISS may comprise at least one modified base. As used herein, the term "modified base" is synonymous with "base analog", for example, "modified cytosine" is synonymous with "cytosine analog." Similarly, "modified" nucleosides or nucleotides are herein defined as being synonymous with nucleoside or nucleotide "analogs." Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the ISS. Preferably, the electron-withdrawing moiety is a halogen. Such modified cytosines can include, but are not limited to, azacytosine, 5-bromocytosine, bromouracil, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, 5-fluorocytosine, fluoropyrimidine, fluorouracil, 5,6-dihydrocytosine, 5-iodocytosine, hydroxyurea, iodouracil, 5-nitrocytosine, uracil, and any other pyrimidine analog or modified pyrimidine.

The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also been described (including, but not limited to, e.g., U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

In some embodiments, an ISS-containing polynucleotide is less than about any of the following lengths (in bases or base pairs): 10,000; 5,000; 2500; 2000; 1500; 1250; 1000; 750; 500; 300; 250; 200; 175; 150; 125; 100; 75; 50; 25; 10. In some embodiments, an ISS-containing polynucleotide is greater than about any of the following lengths (in  
5 bases or base pairs): 8; 10; 15; 20; 25; 30; 40; 50; 60; 75; 100; 125; 150; 175; 200; 250; 300; 350; 400; 500; 750; 1000; 2000; 5000; 7500; 10000; 20000; 50000.

### First Antigen

The invention applies to any antigen, and, for embodiments in which a second  
10 antigen is not co-administered with the first antigen and ISS-containing polynucleotide, is particularly suited to those contexts in which an individual will be exposed to first and second antigens at approximately the same time. The second antigen may or may not be identified, and the first and second antigen may or may not be related, in terms of source. For example, there are a significant number of different grasses, trees and weeds (including  
15 ragweed, in which Amb a 1 is immunodominant and has been the most well-characterized), which cause allergy problems during certain seasons. Using the methods and/or compositions of the invention, Amb a 1 could be administered with the ISS, and an immune response, particularly a Th1 immune response, would be mounted against other antigens which would be expected to be encountered concurrently during ragweed season.  
20 As another example, individuals often have multiple allergies. In this instance, an ISS is administered with any allergen (with respect to the individual) and the individual mounts a Th1 response to other allergen(s). As another example, viruses typically are comprised of proteins which tend to be conserved, and thus relatively constant in sequence (i.e., do not mutate at a frequent rate) and proteins which are variable. In this context, the first antigen  
25 may be a constant polypeptide (such as a core polypeptide or a group- or sub-group specific, internal antigen), which, when administered with an ISS, would modulate an immune response against the virus' variable polypeptides (such as outer coat or type-specific antigens).

In some embodiments, the first antigen is an allergen. Examples of recombinant  
30 allergens are provided in Table 1. Preparation of many allergens is well-known in the art, including, but not limited to, preparation of ragweed pollen allergen Antigen E (*Amb a1*) (Rafnar et al. (1991) *J. Biol. Chem.* 266:1229-1236), major dust mite allergens *Der p1* and

- Der PII* (Chua et al. (1988) *J. Exp. Med.* 167:175-182; Chua et al. (1990) *Int. Arch. Allergy Appl. Immunol.* 91:124-129), white birch pollen *Bet v1* (Breiteneder et al. (1989) *EMBO J.* 8:1935-1938), domestic cat allergen *Fel d1* (Rogers et al. (1993) *Mol. Immunol.* 30:559-568), and protein antigens from tree pollen (Elsayed et al. (1991) *Scand. J. Clin. Lab. Invest. Suppl.* 204:17-31). Preparation of protein antigens from grass pollen for *in vivo* administration has been reported. Malley (1989) *J. Reprod. Immunol.* 16:173-186. Table 1 shows a list of allergens that may be used.

**TABLE 1****RECOMBINANT ALLERGENS**

10

Group	Allergen	Reference
ANIMALS:		
CRUSTACEA		
Shrimp/lobster	tropomyosin Pan s 1	Leung et al. <i>J Allergy Clin Immunol</i> , 1996, 98: 954-61 Leung et al. <i>Mol Mar Biol Biotechnol</i> , 1998, 7:12-20
INSECTS		
Ant	Sol i 2 (venom)	Schmidt et al. <i>J Allergy Clin Immunol.</i> , 1996, 98:82-8
Bee	phospholipase A2 (PLA)  Hyaluronidase (Hya)	Muller et al. <i>J Allergy Clin Immunol</i> , 1995, 96:395-402 Forster et al. <i>J Allergy Clin Immunol</i> , 1995, 95:1229-35 Muller et al. <i>Clin Exp Allergy</i> , 1997, 27:915-20  Soldatova et al. <i>J Allergy Clin Immunol</i> , 1998, 101:691-8
Cockroach	Bla g Bd9OK  Bla g 4 (a calycin)  glutathione S-transferase  Per a 3	Helm et al. <i>J Allergy Clin Immunol</i> , 1996, 98:172-80  Vailes et al. <i>J Allergy Clin Immunol</i> , 1998, 101:274-80  Arruda et al. <i>J Biol Chem</i> , 1997, 272:20907-12  Wu et al. <i>Mol Immunol</i> , 1997, 34:1-8

Group	Allergen	Reference
Dust mite	Der p 2 (major allergen)	Lynch et al. J Allergy Clin Immunol, 1998, 101:562-4 Hakkaart et al. Clin Exp Allergy, 1998, 28:169-74 Hakkaart et al. Clin Exp Allergy, 1998, 28:45-52 Hakkaart et al. Int Arch Allergy Immunol, 1998, 115 (2):150-6 Mueller et al. J Biol Chem, 1997, 272:26893-8
	Der p 2 variant	Smith et al. J Allergy Clin Immunol, 1998, 101:423-5
	Der f 2	Yasue et al. Clin Exp Immunol, 1998, 113:1-9 Yasue et al. Cell Immunol, 1997, 181:30-7
	Der p 10	Asturias et al. Biochim Biophys Acta, 1998, 1397:27-30
	Tyr p 2	Eriksson et al. Eur J Biochem, 1998
Hornet	Antigen 5 aka Dol m V (venom)	Tomalski et al. Arch Insect Biochem Physiol, 1993, 22:303-13
Mosquito	Aed a I (salivary apyrase)	Xu et al. Int Arch Allergy Immunol, 1998, 115:245-51
Yellow jacket	antigen 5, hyaluronidase, and phospholipase (venom)	King et al. J Allergy Clin Immunol, 1996, 98:588-600
<b>MAMMALS</b>		
Cat	Fel d I	Slunt et al. J Allergy Clin Immunol, 1995, 95:1221-8 Hoffmann et al. J Allergy Clin Immunol, 1997, 99: 227-32 Hedlin Curr Opin Pèdiatr, 1995, 7:676-82
Cow	Bos d 2 (dander; a lipocalin)	Zeiler et al. J Allergy Clin Immunol, 1997, 100:721-7 Rautiainen et al. Biochem Bioph. Res Comm., 1998, 247:746-50
	$\beta$ -lactoglobulin (BLG, major cow milk allergen)	Chatel et al. Mol Immunol, 1996, 33:1113-8 Lehrer et al. Crit Rev Food Sci Nutr, 1996, 36:553-64
Dog	Can f 1 and Can f 2, salivary lipocalins	Konieczny et al. Immunology, 1997, 92:577-86 Spitzauer et al. J Allergy Clin Immunol, 1994, 93:614-27 Vrtala et al. J Immunol, 1998, 160:6137-44
Horse	Equ c 1 (major allergen, a lipocalin)	Gregoire et al. J Biol Chem, 1996, 271:32951-9
Mouse	mouse urinary protein (MUP)	Konieczny et al. Immunology, 1997, 92:577-86
<b>OTHER MAMMALIAN ALLERGENS</b>		



Group	Allergen	Reference
Insulin		Ganz et al. J Allergy Clin Immunol, 1990, 86:45-51 Grammer et al. J Lab Clin Med, 1987, 109:141-6 Gonzalo et al. Allergy, 1998, 53:106-7
Interferons	interferon alpha 2c	Detmar et al. Contact Dermatitis, 1989, 20:149-50
MOLLUSCS	topomyosin	Leung et al. J Allergy Clin Immunol, 1996, 98:954-61
PLANT ALLERGENS:		
Barley	Hor v 9	Astwood et al. Adv Exp Med Biol, 1996, 409:269-77
Birch	pollen allergen, Bet v 4 rBet v 1 Bet v 2: (profilin)	Twardosz et al. Biochem Bioph. Res Comm., 1997, 23 9:197  Pauli et al. J Allergy Clin Immunol, 1996, 97:1100-9 van Neerven et al. Clin Exp Allergy, 1998, 28:423-33 Jahn-Schmid et al. Immunotechnology, 1996, 2:103-13 Breitwieser et al. Biotechniques, 1996, 21:918-25 Fuchs et al. J Allergy Clin Immunol, 1997, 100:3 56-64
Brazil nut	globulin	Bartolome et al. Allergol Immunopathol, 1997, 25:135-44
Cherry	Pru a 1 (major allergen)	Scheurer et al. Mol Immunol, 1997, 34:619-29
Corn	Zm13 (pollen)	Heiss et al. FEBS Lett, 1996, 381:217-21 Lehrer et al. Int Arch Allergy Immunol, 1997, 113:122-4
Grass	Phl p 1, Phl p 2, Phl p 5 (timothy grass pollen)	Bufe et al. Am J Respir Crit Care Med, 1998, 157:1269-76 Vrtala et al. J Immunol Jun 15, 1998, 160:6137-44 Niederberger et al. J Allergy Clin Immun., 1998, 101:258-64
	Hol 1 5 velvet grass pollen	Schramm et al. Eur J Biochem, 1998, 252:200-6
	Bluegrass allergen	Zhang et al. J Immunol, 1993, 151:791-9
	Cyn d 7 Bermuda grass	Smith et al. Int Arch Allergy Immunol, 1997, 114:265-71
	Cyn d 12 (a profilin)	Asturias et al. Clin Exp Allergy, 1997, 27:1307-13 Fuchs et al. J Allergy Clin Immunol, 1997, 100:356-64
Juniper	Jun o 2 (pollen)	Tinghino et al. J Allergy Clin Immunol, 1998, 101:772-7
Latex	Hev b 7	Sowka et al. Eur J Biochem, 1998, 255:213-9 Fuchs et al. J Allergy Clin Immunol, 1997, 100:3 56-64
Mercurialis	Mer a 1 (profilin)	Vallverdu et al. J Allergy Clin Immunol, 1998, 101:3 63-70

Group	Allergen	Reference
Mustard (Yellow)	Sin a I (seed)	Gonzalez de la Pena et al. Biochem Bioph. Res Comm., 1993, 190:648-53
Oilseed rape	Bra r I pollen allergen	Smith et al. Int Arch Allergy Immunol, 1997, 114:265-71
Peanut	Ara h I	Stanley et al. Adv Exp Med Biol, 1996, 409:213-6 Burks et al. J Clin Invest, 1995, 96:1715-21 Burks et al. Int Arch Allergy Immunol, 1995, 107:248-50
Poa pratensis	Poa p9	Parronchi et al. Eur J Immunol, 1996, 26:697-703 Astwood et al. Adv Exp Med Biol, 1996, 409:269-77
Ragweed	Amb a I	Sun et al. Biotechnology Aug, 1995, 13:779-86 Hirschwehr et al. J Allergy Clin Immunol, 1998, 101:196-206 Casale et al. J Allergy Clin Immunol, 1997, 100:110-21
Rye	Lol p I	Tamborini et al. Eur J Biochem, 1997, 249:886-94
Walnut	Jug r I	Teuber et al. J Allergy Clin Immunol, 1998, 101:807-14
Wheat	allergen	Fuchs et al. J Allergy Clin Immunol, 1997, 100:356-64 Donovan et al. Electrophoresis, 1993, 14:917-22
<b>FUNGI:</b>		
Aspergillus	Asp f 1, Asp f 2, Asp f3, Asp f 4, rAsp f 6  Manganese superoxide dismutase (MNSOD)	Crameri et al. Mycoses, 1998, 41 Suppl 1:56-60 Hennemann et al. Eur J Immunol, 1998, 28:1155-60 Banerjee et al. J Allergy Clin Immunol, 1997, 99:821-7 Crameri Int Arch Allergy Immunol, 1998, 115:99-114 Crameri et al. Adv Exp Med Biol, 1996, 409:111-6 Moser et al. J Allergy Clin Immunol, 1994, 93: 1-11  Mayer et al. Int Arch Allergy Immunol, 1997, 113:213-5
Blomia	allergen	Caraballo et al. Adv Exp Med Biol, 1996, 409:81-3
Penicillium	allergen	Shen et al. Clin Exp Allergy, 1997, 27:682-90
Psilocybe	Psi c 2	Homer et al. Int Arch Allergy Immunol, 1995, 107:298-300

In some embodiments, the first antigen is a viral conserved, or constant polypeptide, such as a core polypeptide. Administration of an ISS with an antigen comprising a constant polypeptide (which includes constant domain(s), region(s), or fragment(s) of a constant polypeptide) results in a Th1-type response to the viral antigen with the constant domain. Upon viral infection and exposure to all the viral antigens, an immune response to the

constant domain antigen as well as to other antigens, including those which vary between strains, is mounted.

Conserved polypeptides include, but are not limited to, core proteins such as HIV gag proteins (including, but not limited to, membrane anchoring (MA) protein, core capsid (CA) protein and nucleocapsid (NC) protein), HIV polymerase, influenza virus matrix (M) protein and influenza virus nucleocapsid (NP) protein. References discussing influenza vaccination include Scherle and Gerhard (1988) *Proc. Natl. Acad. Sci. USA* 85:4446-4450; Scherle and Gerhard (1986) *J. Exp. Med.* 164:1114-1128; Granoff et al. (1993) *Vaccine* 11:S46-51; Kodihalli et al. (1997) *J. Virol.* 71:3391-3396; Ahmeida et al. (1993) *Vaccine* 11:1302-1309; Chen et al. (1999) *Vaccine* 17:653-659; Govorkova and Smimov (1997) *Acta Virol.* (1997) 41:251-257; Koide et al. (1995) *Vaccine* 13:3-5; Mbawuike et al. (1994) *Vaccine* 12:1340-1348; Tamura et al. (1994) *Vaccine* 12:310-316; Tamura et al. (1992) *Eur. J. Immunol.* 22:477-481; Hirabayashi et al. (1990) *Vaccine* 8:595-599. Other examples of conserved polypeptides are group- or sub-group specific antigens, which are known for a number of infectious agents, including, but not limited to, adenovirus, herpes simplex virus, papilloma virus, respiratory syncytial virus and poxviruses.

In some embodiments, the first antigen is linked to or associated with a carrier molecule. Typically, such an antigen is conjugated to the carrier molecule. In other embodiments, first antigen is a carrier molecule. In embodiments comprising a carrier molecule, the ISS containing polynucleotide may or may not be proximately associated (such as by conjugation) to the carrier molecule. In some embodiments, both the first antigen and the ISS are conjugated to the carrier molecule.

Carriers are known in the art. Plotkin, *Vaccines* 3rd Ed. Philadelphia, WB Saunders Co. (1999). Bacterial carriers (i.e., carriers derived from bacteria) include, but are not limited to, cholera toxin B subunit (CTB); diphtheria toxin mutant (CRM197); diphtheria toxoid; group B streptococcus alpha C protein; meningococcal outer membrane protein (OMPC); tetanus toxoid; outer membrane protein of nontypeable *Haemophilus influenza* (such as P6); recombinant class 3 porin (rPorB) of group B meningococci; heat-killed *Brucella abortus*; heat-killed *Listeria monocytogenes*; and *Pseudomonas aeruginosa* recombinant exoprotein A. Another carrier is keyhole limpet hemocyanin (KLH). Examples of viral-derived carriers are known in the art and include hepatitis b surface

antigen (HBsAg) particles and hepatitis b core antigen (HBcAg). In some embodiments, the first antigen comprises a viral vector, such as vaccinia, adenovirus, and canary pox.

Many antigenic peptides and proteins are known, and available in the art; others can be identified using conventional techniques. For immunization against tumor formation, immunomodulatory peptides can include tumor cells (live or irradiated), tumor cell  
5 extracts, or protein subunits of tumor antigens such as Her-2/neu, Mart1, carcinoembryonic antigen (CEA), gangliosides, human milk fat globule (HMFG), mucin (MUC1), MAGE antigens, BAGE antigens, GAGE antigens, gp100, prostate specific antigen (PSA), and tyrosinase. Vaccines for immuno-based contraception can be formed by including sperm  
10 proteins administered with ISS. Lea et al. (1996) *Biochim. Biophys. Acta* 1307:263.

Attenuated and inactivated viruses are suitable for use herein as the antigen. Preparation of these viruses is well-known in the art and many are commercially available (see, e.g., Physicians' Desk Reference (1998) 52nd edition, Medical Economics Company, Inc.). For example, polio virus is available as IPOL® (Pasteur Merieux Connaught) and  
15 ORIMUNE® (Lederle Laboratories), hepatitis A virus as VAQTA® (Merck), measles virus as ATTENUVAX® (Merck), mumps virus as MUMPSVAX® (Merck) and rubella virus as MERUVAX®II (Merck). Additionally, attenuated and inactivated viruses such as HIV-1, HIV-2, herpes simplex virus, hepatitis B virus, rotavirus, human and non-human papillomavirus and slow brain viruses can provide peptide antigens.

In some embodiments, the first antigen is an antigen from an infectious agent, including protozoan, bacterial, fungal (including unicellular and multicellular), and viral  
20 infectious agents. Examples of suitable viral antigens have been described above. Bacteria include *Hemophilus influenza*, *Mycobacterium tuberculosis* and *Bordetella pertussis*. Protozoan infectious agents include malarial plasmodia, Leishmania species, Trypanosoma species and Schistosoma species. Fungi include *Candida albicans*.  
25

Antigens may be isolated from their source using purification techniques known in the art or, more conveniently, may be produced using recombinant methods.

Antigenic peptides can include purified native peptides, synthetic peptides, recombinant proteins, crude protein extracts, attenuated or inactivated viruses, cells, micro-  
30 organisms, or fragments of such peptides. Immunomodulatory peptides can be native or synthesized chemically or enzymatically. Any method of chemical synthesis known in the art is suitable. Solution phase peptide synthesis can be used to construct peptides of

moderate size or, for the chemical construction of peptides, solid phase synthesis can be employed. Atherton et al. (1981) *Hoppe Seylers Z. Physiol. Chem.* 362:833-839. Proteolytic enzymes can also be utilized to couple amino acids to produce peptides. Kullmann (1987) *Enzymatic Peptide Synthesis*, CRC Press, Inc. Alternatively, the peptide  
5 can be obtained by using the biochemical machinery of a cell, or by isolation from a biological source. Recombinant DNA techniques can be employed for the production of peptides. Hames et al. (1987) *Transcription and Translation: A Practical Approach*, IRL Press. Peptides can also be isolated using standard techniques such as affinity chromatography.

10 Preferably the antigens are peptides, lipids (e.g. sterols, fatty acids, and phospholipids), polysaccharides such as those used in *H. influenza* vaccines, gangliosides and glycoproteins. These can be obtained through several methods known in the art, including isolation and synthesis using chemical and enzymatic methods. In certain cases, such as for many sterols, fatty acids and phospholipids, the antigenic portions of the  
15 molecules are commercially available.

### Second Antigen

In some embodiments, a second antigen is administered with the ISS-containing polynucleotide which is proximately associated with a first antigen. A second antigen may  
20 be any antigen other than the first antigen, and can be different antigenic regions from the same polypeptide. A second antigen may be any of the antigens described herein, and the principles of obtaining and/or isolating such antigens likewise apply in the context of "second" antigens. Other examples of second antigens are viral variable polypeptides (which includes variable regions or domains of polypeptides), such as coat proteins,  
25 including influenza HA or NA. In embodiments which entail administering second antigen, if a first antigen is a conserved polypeptide, the second antigen is generally a variable polypeptide. For example, an influenza vaccine formulation is administered comprising coat protein(s) and core polypeptide such as NP with an ISS-containing polynucleotide. As another example, an HIV vaccine formulation containing a gp120  
30 polypeptide and core antigen with ISS-containing polynucleotide is administered.

### ISS-Antigen

ISS may be administered with first antigen in a number of ways. For embodiments which entail administration of first antigen without administration of second antigen, an ISS-containing polynucleotide and first antigen may be administered spatially proximate with respect to each other, or as an admixture (i.e., in solution). For embodiments which entail administration of a second antigen in addition to a first antigen, an ISS-containing polynucleotide is spatially proximate to the first antigen. As described below, spatial proximation can be accomplished in a number of ways, including conjugation, encapsidation, via affixation to a platform or adsorption onto a surface. Generally, and most preferably, an ISS-containing polynucleotide and first antigen are proximately associated at a distance effective to enhance the immune response generated compared to the administration of the ISS and first antigen as an admixture.

The ISS portion can be coupled with the antigen portion of a conjugate in a variety of ways, including covalent and/or non-covalent interactions.

The link between the portions can be made at the 3' or 5' end of the ISS, or at a suitably modified base at an internal position in the ISS. If the antigen is a peptide and contains a suitable reactive group (e.g., an N-hydroxysuccinimide ester) it can be reacted directly with the N<sup>4</sup> amino group of cytosine residues. Depending on the number and location of cytosine residues in the ISS, specific coupling at one or more residues can be achieved.

Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the ISS. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, the antigen of interest.

Where the antigen is a peptide, this portion of the conjugate can be attached to the 3'-end of the ISS through solid support chemistry. For example, the ISS portion can be added to a polypeptide portion that has been pre-synthesized on a support. Haralambidis et al. (1990a) *Nucleic Acids Res.* 18:493-499; and Haralambidis et al. (1990b) *Nucleic Acids Res.* 18:501-505. Alternatively, the ISS can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end. Upon chemical cleavage of the ISS from the support, a terminal thiol group is left at the 3'-end of the oligonucleotide (Zuckermann et al. (1987) *Nucleic Acids Res.* 15:5305-5321; and Corey et

al. (1987) *Science* 238:1401-1403) or a terminal amino group is left at the 3'-end of the oligonucleotide (Nelson et al. (1989) *Nucleic Acids Res.* 17:1781-1794). Conjugation of the amino-modified ISS to amino groups of the peptide can be performed as described in Benoit et al. (1987) *Neuromethods* 6:43-72. Conjugation of the thiol-modified ISS to  
5 carboxyl groups of the peptide can be performed as described in Sinah et al. (1991) *Oligonucleotide Analogues: A Practical Approach*, IRL Press. Coupling of an oligonucleotide carrying an appended maleimide to the thiol side chain of a cysteine residue of a peptide has also been described. Tung et al. (1991) *Bioconjug. Chem.* 2:464-465.

10 The peptide portion of the conjugate can be attached to the 5'-end of the ISS through an amine, thiol, or carboxyl group that has been incorporated into the oligonucleotide during its synthesis. Preferably, while the oligonucleotide is fixed to the solid support, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite at the other, is covalently attached to the 5'-hydroxyl. Agrawal et  
15 al. (1986) *Nucleic Acids Res.* 14:6227-6245; Connolly (1985) *Nucleic Acids Res.* 13:4485-4502; Kremsky et al. (1987) *Nucleic Acids Res.* 15:2891-2909; Connolly (1987) *Nucleic Acids Res.* 15:3131-3139; Bischoff et al. (1987) *Anal. Biochem.* 164:336-344; Blanks et al. (1988) *Nucleic Acids Res.* 16:10283-10299; and U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802. Subsequent to deprotection, the amine, thiol, and carboxyl  
20 functionalities can be used to covalently attach the oligonucleotide to a peptide. Benoit et al. (1987); and Sinah et al. (1991).

An ISS-antigen conjugate can also be formed through non-covalent interactions, such as ionic bonds, hydrophobic interactions, hydrogen bonds and/or van der Waals attractions.

25 Non-covalently linked conjugates can include a non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an ISS. Roget et al. (1989) *Nucleic Acids Res.* 17:7643-7651. Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated oligonucleotide.

30 Non-covalent associations can also occur through ionic interactions involving an ISS and residues within the antigen, such as charged amino acids, or through the use of a linker portion comprising charged residues that can interact with both the oligonucleotide

and the antigen. For example, non-covalent conjugation can occur between a generally negatively-charged ISS and positively-charged amino acid residues of a peptide, e.g., polylysine, polyarginine and polyhistidine residues.

Non-covalent conjugation between ISS and antigens can occur through DNA binding motifs of molecules that interact with DNA as their natural ligands. For example, such DNA binding motifs can be found in transcription factors and anti-DNA antibodies.

The linkage of the ISS to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al. (1988) *Nucleic Acids Symp. Ser.* 19:189-192), oligonucleotide-fatty acid conjugates (Grabarek et al. (1990) *Anal. Biochem.* 185:131-135; and Staros et al. (1986) *Anal. Biochem.* 156:220-222), and oligonucleotide-sterol conjugates. Boujrad et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5728-5731.

The linkage of the oligonucleotide to an oligosaccharide can be formed using standard known methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin. O'Shannessy et al. (1985) *J. Applied Biochem.* 7:347-355.

The linkage of a circular ISS to a peptide or antigen can be formed in several ways. Where the circular ISS is synthesized using recombinant or chemical methods, a modified nucleoside is suitable. Ruth (1991) in *Oligonucleotides and Analogues: A Practical Approach*, IRL Press. Standard linking technology can then be used to connect the circular ISS to the antigen or other peptide. Goodchild (1990) *Bioconjug. Chem.* 1:165. Where the circular ISS is isolated, or synthesized using recombinant or chemical methods, the linkage can be formed by chemically activating, or photoactivating, a reactive group (e.g. carbene, radical) that has been incorporated into the antigen or other peptide.

Additional methods for the attachment of peptides and other molecules to oligonucleotides can be found in U.S. Patent No. 5,391,723; Kessler (1992) "Nonradioactive labeling methods for nucleic acids" in Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press; and Geoghegan et al. (1992) *Bioconjug. Chem.* 3:138-146.

An ISS may be proximately associated with an antigen(s) in other ways. In some embodiments, an ISS and antigen are proximately associated by encapsulation. In other embodiments, an ISS and antigen are proximately associated by linkage to a platform



molecule. A "platform molecule" (also termed "platform") is a molecule containing sites which allow for attachment of the ISS and antigen(s). In other embodiments, an ISS and antigen are proximately associated by adsorption onto a surface, preferably a carrier particle.

5 In some embodiments, the methods of the invention employ an encapsulating agent that can maintain the proximate association of the ISS and first antigen until the complex is available to the target (or compositions comprising such encapsulating agents). Preferably, the composition comprising ISS, antigen and encapsulating agent is in the form of adjuvant oil-in-water emulsions, microparticles and/or liposomes. More preferably, adjuvant oil-in-  
10 water emulsions, microparticles and/or liposomes encapsulating an ISS-immunomodulatory molecule are in the form of particles from about 0.04  $\mu\text{m}$  to about 100  $\mu\text{m}$  in size, preferably any of the following ranges: from about 0.1  $\mu\text{m}$  to about 20  $\mu\text{m}$ ; from about 0.15  $\mu\text{m}$  to about 10  $\mu\text{m}$ ; from about 0.05  $\mu\text{m}$  to about 1.00  $\mu\text{m}$ ; from about 0.05  $\mu\text{m}$  to about 0.5  $\mu\text{m}$ .

15 Colloidal dispersion systems, such as microspheres, beads, macromolecular complexes, nanocapsules and lipid-based system, such as oil-in-water emulsions, micelles, mixed micelles and liposomes can provide effective encapsulation of ISS-containing compositions.

The encapsulation composition further comprises any of a wide variety of  
20 components. These include, but are not limited to, alum, lipids, phospholipids, lipid membrane structures (LMS), polyethylene glycol (PEG) and other polymers, such as polypeptides, glycopeptides, and polysaccharides.

Polypeptides suitable for encapsulation components include any known in the art and include, but are not limited to, fatty acid binding proteins. Modified polypeptides  
25 contain any of a variety of modifications, including, but not limited to glycosylation, phosphorylation, myristylation, sulfation and hydroxylation. As used herein, a suitable polypeptide is one that will protect an ISS-containing composition to preserve the immunomodulatory activity thereof. Examples of binding proteins include, but are not limited to, albumins such as bovine serum albumin (BSA) and pea albumin.

30 Other suitable polymers can be any known in the art of pharmaceuticals and include, but are not limited to, naturally-occurring polymers such as dextrans, hydroxyethyl starch, and polysaccharides, and synthetic polymers. Examples of naturally occurring

polymers include proteins, glycopeptides, polysaccharides, dextran and lipids. The additional polymer can be a synthetic polymer. Examples of synthetic polymers which are suitable for use in the present invention include, but are not limited to, polyalkyl glycols (PAG) such as PEG, polyoxyethylated polyols (POP), such as polyoxyethylated glycerol (POG), polytrimethylene glycol (PTG) polypropylene glycol (PPG), polyhydroxyethyl methacrylate, polyvinyl alcohol (PVA), polyacrylic acid, polyethyloxazoline, polyacrylamide, polyvinylpyrrolidone (PVP), polyamino acids, polyurethane and polyphosphazene. The synthetic polymers can also be linear or branched, substituted or unsubstituted, homopolymeric, co-polymers, or block co-polymers of two or more different synthetic monomers.

The PEGs for use in encapsulation compositions of the present invention are either purchased from chemical suppliers or synthesized using techniques known to those of skill in the art.

The term "LMS", as used herein, means lamellar lipid particles wherein polar head groups of a polar lipid are arranged to face an aqueous phase of an interface to form membrane structures. Examples of the LMSs include liposomes, micelles, cochleates (i.e., generally cylindrical liposomes), microemulsions, unilamellar vesicles, multilamellar vesicles, and the like.

A preferred colloidal dispersion system of this invention is a liposome. In mice immunized with a liposome-encapsulated antigen, liposomes appeared to enhance a Th1-type immune response to the antigen. Aramaki et al. (1995) *Vaccine* 13:1809-1814. As used herein, a "liposome" or "lipid vesicle" is a small vesicle bounded by at least one and possibly more than one bilayer lipid membrane. Liposomes are made artificially from phospholipids, glycolipids, lipids, steroids such as cholesterol, related molecules, or a combination thereof by any technique known in the art, including but not limited to sonication, extrusion, or removal of detergent from lipid-detergent complexes. A liposome can also optionally comprise additional components, such as a tissue targeting component. It is understood that a "lipid membrane" or "lipid bilayer" need not consist exclusively of lipids, but can additionally contain any suitable other components, including, but not limited to, cholesterol and other steroids, lipid-soluble chemicals, proteins of any length, and other amphipathic molecules, providing the general structure of the membrane is a sheet of two hydrophilic surfaces sandwiching a hydrophobic core. For a general

discussion of membrane structure, see *The Encyclopedia of Molecular Biology* by J. Kendrew (1994). For suitable lipids see e.g., Lasic (1993) "Liposomes: from Physics to Applications" Elsevier, Amsterdam.

Processes for preparing liposomes containing ISS-containing compositions are known in the art. The lipid vesicles can be prepared by any suitable technique known in the art. Methods include, but are not limited to, microencapsulation, microfluidization, LLC method, ethanol injection, freon injection, the "bubble" method, detergent dialysis, hydration, sonication, and reverse-phase evaporation. Reviewed in Watwe et al. (1995) *Curr. Sci.* 68:715-724. Techniques may be combined in order to provide vesicles with the most desirable attributes.

The invention encompasses use of LMSs containing tissue or cellular targeting components. Such targeting components are components of a LMS that enhance its accumulation at certain tissue or cellular sites in preference to other tissue or cellular sites when administered to an intact animal, organ, or cell culture. A targeting component is generally accessible from outside the liposome, and is therefore preferably either bound to the outer surface or inserted into the outer lipid bilayer. A targeting component can be *inter alia* a peptide, a region of a larger peptide, an antibody specific for a cell surface molecule or marker, or antigen binding fragment thereof, a nucleic acid, a carbohydrate, a region of a complex carbohydrate, a special lipid, or a small molecule such as a drug, hormone, or hapten, attached to any of the aforementioned molecules. Antibodies with specificity toward cell type-specific cell surface markers are known in the art and are readily prepared by methods known in the art.

The LMSs can be targeted to any cell type toward which a therapeutic treatment is to be directed, e.g., a cell type which can modulate and/or participate in an immune response. Such target cells and organs include, but are not limited to, APCs, such as macrophages, dendritic cells and lymphocytes, lymphatic structures, such as lymph nodes and the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found.

The LMS compositions of the present invention can additionally comprise surfactants. Surfactants can be cationic, anionic, amphiphilic, or nonionic. A preferred class of surfactants are nonionic surfactants; particularly preferred are those that are water soluble.

In embodiments in which an ISS and antigen are proximately associated by linkage to a platform molecule, the platform may be proteinaceous or non-proteinaceous (i.e., organic). Examples of proteinaceous platforms include, but are not limited to, albumin, gammaglobulin, immunoglobulin (IgG) and ovalbumin. Borel et al. (1990) *Immunol. Methods* 126:159-168; Dumas et al. (1995) *Arch. Dermatol. Res.* 287:123-128; Borel et al. (1995) *Int. Arch. Allergy Immunol.* 107:264-267; Borel et al. (1996) *Ann. N.Y. Acad. Sci.* 778:80-87. A platform is multi-valent (i.e., contains more than one binding, or linking, site) to accommodate binding to both an ISS and antigen. Other examples of polymeric platforms are dextran, polyacrylamide, ficoll, carboxymethylcellulose, polyvinyl alcohol, and poly D-glutamic acid/D-lysine.

The principles of using platform molecules are well understood in the art. Generally, a platform contains, or is derivatized to contain, appropriate binding sites for ISS and antigen. In addition, or alternatively, ISS and/or antigen is derivatized to provide appropriate linkage groups. For example, a simple platform is a bi-functional linker (i.e., has two binding sites), such as a peptide. Further examples are discussed below.

Platform molecules may be biologically stabilized, i.e., they exhibit an *in vivo* excretion half-life often of hours to days to months to confer therapeutic efficacy, and are preferably composed of a synthetic single chain of defined composition. They generally have a molecular weight in the range of about 200 to about 200,000, preferably about 200 to about 50,000 (or less, such as 30,000). Examples of valency platform molecules are polymers (or are comprised of polymers) such as polyethylene glycol (PEG; preferably having a molecular weight of about 200 to about 8000), poly-D-lysine, polyvinyl alcohol, polyvinylpyrrolidone, D-glutamic acid and D-lysine (in a ratio of 3:2). Other molecules that may be used are albumin and IgG.

Other platform molecules suitable for use within the present invention are the chemically-defined, non-polymeric valency platform molecules disclosed in U.S. patent 5,552,391. Other homogeneous chemically-defined valency platform molecules suitable for use within the present invention are derivatized 2,2'-ethylenedioxydiethylamine (EDDA) and triethylene glycol (TEG).

Additional suitable valency platform molecules include, but are not limited to, tetraaminobenzene, heptaaminobetacyclodextrin, tetraaminopentaerythritol, 1,4,8,11-tetraazacyclotetradecane (Cyclam) and 1,4,7,10-tetraazacyclododecane (Cyclen).

In general, these platforms are made by standard chemical synthesis techniques. PEG must be derivatized and made multivalent, which is accomplished using standard techniques. Some substances suitable for conjugate synthesis, such as PEG, albumin, and IgG are available commercially.

5           Conjugation of an ISS and antigen to a platform molecule may be effected in any number of ways, typically involving one or more crosslinking agents and functional groups on the antigen and ISS platform and platform molecule. Platforms and ISS and antigen must have appropriate linking groups. Linking groups are added to platforms using standard synthetic chemistry techniques. Linking groups may be added to polypeptide  
10           antigens and ISS using either standard solid phase synthetic techniques or recombinant techniques. Recombinant approaches may require post-translational modification in order to attach a linker, and such methods are known in the art.

          As an example, polypeptides contain amino acid side chain moieties containing functional groups such as amino, carboxyl or sulfhydryl groups that serve as sites for  
15           coupling the polypeptide to the platform. Residues that have such functional groups may be added to the polypeptide if the polypeptide does not already contain these groups. Such residues may be incorporated by solid phase synthesis techniques or recombinant techniques, both of which are well known in the peptide synthesis arts. When the polypeptide has a carbohydrate side chain(s) (or if the antigen is a carbohydrate), functional  
20           amino, sulfhydryl and/or aldehyde groups may be incorporated therein by conventional chemistry. For instance, primary amino groups may be incorporated by reaction with ethylenediamine in the presence of sodium cyanoborohydride, sulfhydryls may be introduced by reaction of cysteamine dihydrochloride followed by reduction with a standard disulfide reducing agent, while aldehyde groups may be generated following  
25           periodate oxidation. In a similar fashion, the platform molecule may also be derivatized to contain functional groups if it does not already possess appropriate functional groups.

          Hydrophilic linkers of variable lengths are useful for connecting ISS and antigen to platform molecules. Suitable linkers include linear oligomers or polymers of ethylene glycol. Such linkers include linkers with the formula

30            $R^1S(CH_2CH_2O)_nCH_2CH_2O(CH_2)_mCO_2R^2$  wherein  $n = 0-200$ ,  $m = 1$  or  $2$ ,  $R^1 = H$  or a protecting group such as trityl,  $R^2 = H$  or alkyl or aryl, e.g., 4-nitrophenyl ester. These linkers are useful in connecting a molecule containing a thiol reactive group such as

haloacetyl, maleiamide, etc., via a thioether to a second molecule which contains an amino group via an amide bond. These linkers are flexible with regard to the order of attachment, *i.e.*, the thioether can be formed first or last.

5 In embodiments in which an ISS and antigen are proximately associated by adsorption onto a surface, the surface may be in the form of a carrier particle (for example, a nanoparticle) made with either an inorganic or organic core. Examples of such nanoparticles include, but are not limited to, nanocrystalline particles, nanoparticles made by the polymerization of alkylcyanoacrylates and nanoparticles made by the polymerization of methyldiene malonate. Additional surfaces to which an ISS and antigen may be  
10 adsorbed include, but are not limited to, activated carbon particles and protein-ceramic nanoplates.

Adsorption of polynucleotides and polypeptides to a surface for the purpose of delivery of the adsorbed molecules to cells is well known in the art. See, for example, Douglas et al. (1987) *Crit. Rev. Ther. Drug. Carrier Syst.* 3:233-261; Hagiwara et al.  
15 (1987) *In Vivo* 1:241-252; Bousquet et al. (1999) *Pharm. Res.* 16:141-147; and Kossovsky et al., U.S. Patent 5,460,831. Preferably, the material comprising the adsorbent surface is biodegradable. Adsorption of an ISS and/or antigen to a surface may occur through non-covalent interactions, including ionic and/or hydrophobic interactions.

In general, characteristics of nanoparticles, such as surface charge, particle size and  
20 molecular weight, depend upon polymerization conditions, monomer concentration and the presence of stabilizers during the polymerization process (Douglas et al., 1987). The surface of carrier particles may be modified, for example, with a surface coating, to allow or enhance adsorption of the ISS and/or antigen. Carrier particles with adsorbed ISS and/or antigen may be further coated with other substances. The addition of such other substances  
25 may, for example, prolong the half-life of the particles once administered to the subject and/or may target the particles to a specific cell type or tissue, as described herein.

Nanocrystalline surfaces to which an ISS and antigen may be adsorbed have been described (see, for example, U.S. Patent 5,460,831). Nanocrystalline core particles (with  
30 diameters of 1  $\mu\text{m}$  or less) are coated with a surface energy modifying layer that promotes adsorption of polypeptides, polynucleotides and/or other pharmaceutical agents. As described in U.S. Patent 5,460,831, for example, a core particle is coated with a surface that promotes adsorption of an oligonucleotide and is subsequently coated with an antigen

preparation, for example, in the form of a lipid-antigen mixture. Such nanoparticles are self-assembling complexes of nanometer sized particles, typically on the order of 0.1  $\mu\text{m}$ , that carry an inner layer of ISS and an outer layer of antigen.

Another adsorbent surface are nanoparticles made by the polymerization of alkylcyanoacrylates. Alkylcyanoacrylates can be polymerized in acidified aqueous media by a process of anionic polymerization. Depending on the polymerization conditions, the small particles tend to have sizes in the range of 20 to 3000 nm, and it is possible to make nanoparticles specific surface characteristics and with specific surface charges (Douglas et al., 1987). For example, oligonucleotides may be adsorbed to polyisobutyl- and polyisohexylcyanoacrylate nanoparticles in the presence of hydrophobic cations such as tetraphenylphosphonium chloride or quaternary ammonium salts, such as cetyltrimethyl ammonium bromide. Oligonucleotide adsorption on these nanoparticles appears to be mediated by the formation of ion pairs between negatively charged phosphate groups of the nucleic acid chain and the hydrophobic cations. See, for example, Lambert et al. (1998) *Biochimie* 80:969-976, Chavany et al. (1994) *Pharm. Res.* 11:1370-1378; Chavany et al. (1992) *Pharm. Res.* 9:441-449. Polypeptides may also be adsorbed to polyalkylcyanoacrylate nanoparticles. See, for example, Douglas et al., 1987; Schroeder et al. (1998) *Peptides* 19:777-780.

Another adsorbent surface are nanoparticles made by the polymerization of methyldiene malonate. For example, as described in Bousquet et al., 1999, polypeptides adsorbed to poly(methyldiene malonate 2.1.2) nanoparticles appear to do so initially through electrostatic forces followed by stabilization through hydrophobic forces.

#### Administration and Assessment of the Immune Response

The ISS-containing polynucleotide and first antigen can be administered in combination with other pharmaceutical and/or immunogenic and/or immunostimulatory agents and can be combined with a physiologically acceptable carrier thereof. The effective amount and method of administration of the particular ISS-first antigen formulation can vary based on the individual, what condition is to be treated and other factors evident to one skilled in the art. A suitable dosage range is one that provides the desired modulation of immune response to second antigen. Generally, if the ISS-containing polynucleotide and first antigen are administered in proximate association to

each other (such as in a conjugate form), a dosage range of the ISS-antigen composition may be, for example, from about any of the following: 1 to 500  $\mu\text{g}$ , 100 to 400  $\mu\text{g}$ , 200 to 300  $\mu\text{g}$ , 1 to 100  $\mu\text{g}$ , 100 to 200  $\mu\text{g}$ , 300 to 400  $\mu\text{g}$ , 400 to 500  $\mu\text{g}$ . In these compositions, the molar ratio of ISS-containing polynucleotide to antigen may vary. If the ISS-containing polynucleotide and first antigen are not proximately associated, i.e., administered as an admixture, generally the dosage ranges are higher, such as, for the ISS-containing polynucleotide, for example, from about any of the following: 10 to 10,000  $\mu\text{g}$ , 2000 to 8000  $\mu\text{g}$ , 4000 to 6000  $\mu\text{g}$ , 10 to 500  $\mu\text{g}$ , 500 to 1000  $\mu\text{g}$ , 1000 to 2000  $\mu\text{g}$ , 2000 to 3000  $\mu\text{g}$ , 6000 to 7000  $\mu\text{g}$ , 7000 to 8000  $\mu\text{g}$ , 8000 to 9000  $\mu\text{g}$ , 9000 to 10,000  $\mu\text{g}$ ; and for the first antigen, for example, from about any of the following: 0.1 to 500  $\mu\text{g}$ , 1.0 to 100  $\mu\text{g}$ , 5 to 50  $\mu\text{g}$ , 0.1 to 1.0  $\mu\text{g}$ , 1.0 to 10  $\mu\text{g}$ , 50 to 200  $\mu\text{g}$ , 200 to 400  $\mu\text{g}$ , 300 to 500  $\mu\text{g}$ . The absolute amount given to each patient depends on pharmacological properties such as bioavailability, clearance rate and route of administration.

*Timing of administration and exposure to second antigen*

Timing of administration and exposure to second antigen encompasses several aspects: first, timing of administration of ISS-containing polynucleotide with respect to first antigen (i.e., whether the first antigen and ISS-containing polynucleotide are administered together or separately); second, timing of administration of ISS-containing polynucleotide and first antigen with respect to exposure to the second antigen; third, timing of exposure of the second antigen with respect to exposure to first antigen; fourth, for those embodiments in which the second antigen is administered with the first antigen, timing of administration of first antigen with respect to second antigen.

Regarding timing of administration of ISS with respect to first antigen, ISS is preferably administered at the same time as administration of first antigen (i.e., is co-administered). Co-administration can occur as a result of administering an admixture of ISS-containing polynucleotide and first antigen or administering ISS-containing polynucleotide in proximate association with first antigen. However, it is contemplated that ISS and first antigen may also be administered within about any of the following (with respect to each other): 14 days, 12 days, 10 days, 7 days, 5 days, 3 days, 2 days, 1 day, 20 hours, 15 hours, 10 hours, 8 hours, 5 hours, 3 hours, 2 hours, 1 hour, 30 minutes, 15 minutes. If ISS and first antigen are not co-administered, preferably ISS is administered before administration of first antigen. The principles described in this paragraph also apply



to timing of administering second antigen in addition to first antigen; thus, the second antigen may be administered within about any of the above times and, in addition, within about any of the following times: 3 weeks, 4 weeks, 6 weeks.

Regarding timing of administering ISS-containing polynucleotide and first antigen with respect to exposure to second antigen, generally, ISS and first antigen are administered either before, during or after exposure to second antigen. If administered before exposure to second antigen, the ISS and first antigen should be administered at an appropriate time before exposure, that is, administration should occur such that the immunomodulation will occur upon exposure to the second antigen. The timing of administration can be determined empirically, for example by measuring any suitable indicia of an antigen-specific immune response as described herein.

Administration of ISS-containing polynucleotide and first antigen occurs at any of various times before exposure to the second antigen, as well as simultaneously with exposure to second antigen. Administration may occur less than about any one or more of the following (with respect to exposure to second antigen): 1 day, 2 days, 3 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 6 weeks, 2 months, 10 weeks, 3 months, 6 months. Additionally, administration may occur more than (at least) about any one or more of the following (with respect to exposure to second antigen): 1 day, 2 days, 3 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 6 weeks, 2 months, 10 weeks, 3 months, 6 months, 1 year, 2 years, 5 years. Administration may consist of one or more doses, at various times. In some embodiments, exposure to second antigen may be at least about any of 1 year, 2 years, 3 years, 5 years, and/or 10 years after administration of ISS-containing polynucleotide and first antigen.

For those embodiments which do not entail administration of a second antigen (and thus require concurrent exposure to first and second antigen), a second antigen may be encountered within a number of hours, days, or even weeks of encountering the first antigen. Accordingly, the first and second antigens may be encountered within about any of the following (with respect to each other): 30 minutes, 1 hour, 2 hours, 5 hours, 12 hours, 20 hours, 24 hours, 2 days, 5 days, 7 days, 10 days, 14 days, 20 days.

#### *Formulations and routes of administration*

Compositions suitable for topical application may be used, including, but not limited to, physiologically acceptable ointments, creams, rinses, sprays and gels. Topical

administration is, for instance, by a dressing or bandage having dispersed therein a delivery system, or by direct administration of a delivery system into incisions or open wounds. Creams, rinses, gels or ointments having dispersed therein an ISS-containing composition are suitable for use as topical ointments or wound filling agents.

5           ISS and first antigen (with or without second antigen) can be administered in conjunction with one or more immunomodulatory facilitators. The ISS and facilitator can be administered in proximate association, such as an ISS-facilitator conjugate and/or they can be co-administered as a complex in the form of an admixture, such as in an emulsion. Immunomodulatory facilitators include, but are not limited to, co-stimulatory molecules  
10 (such as cytokines, chemokines, targeting protein ligand, trans-activating factors, peptides, and peptides comprising a modified amino acid) and adjuvants (such as alum, lipid emulsions, and polylactide/polyglycolide microparticles).

          Among suitable immunomodulatory cytokine peptides for administration with ISS are the interleukins (e.g., IL-1, IL-2, IL-3, etc.), interferons (e.g., IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ ),  
15 erythropoietin, colony stimulating factors (e.g., G-CSF, M-CSF, GM-CSF) and TNF- $\alpha$ . Preferably, immunostimulatory peptides for use in conjunction with ISS oligonucleotides are those that stimulate Th1-type immune responses, such as IL-12 (Bliss et al. (1996) *J. Immunol.* 156:887-894), IL-18, TNF- $\alpha$ ,  $\beta$  and  $\gamma$ , and/or transforming growth factor (TGF)- $\alpha$ .

20           Peptides administered with ISS can also include amino acid sequences that mediate protein binding to a specific receptor or that mediate targeting to a specific cell type or tissue. Examples include, but are not limited to, antibodies or antibody fragments, peptide hormones such as human growth hormone, and enzymes. Immunomodulatory peptides also include peptide hormones, peptide neurotransmitters and peptide growth factors. Co-  
25 stimulatory molecules such as B7 (CD80), trans-activating proteins such as transcription factors, chemokines such as macrophage chemotactic protein (MCP) and other chemoattractant or chemotactic peptides are also useful peptides for administration with ISS.

          An ISS-containing polynucleotide may also be administered in conjunction with an  
30 adjuvant. Administration of an antigen with an ISS and an adjuvant leads to a potentiation of a immune response to the antigen and thus, can result in an enhanced immune response compared to that which results from a composition comprising the ISS and antigen alone.

Adjuvants are known in the art and include, but are not limited to, oil-in-water emulsions, water-in oil emulsions, alum (aluminum salts), liposomes and microparticles, including but not limited to, polystyrene, starch, polyphosphazene and polylactide/polyglycosides. Other suitable adjuvants also include, but are not limited to, MF59, DETOX™ (Ribi), squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) *Nature* 344:873-875, as well as, lipid-based adjuvants and others described herein.

For veterinary use and for production of antibodies in animals, mitogenic components of Freund's adjuvant (both complete and incomplete) can be used.

As with all immunogenic compositions, the immunologically effective amounts of the components must be determined empirically. Factors to be considered include the antigenicity, whether or not ISS and/or antigen will be complexed with or covalently attached to an immunomodulatory facilitator, an adjuvant or carrier protein or other carrier, route of administration and the number of immunizing doses to be administered. Such factors are known in the vaccine art and it is well within the skill in the art to make such determinations.

The route(s) of administration useful in a particular application are apparent to one of skill in the art. Routes of ISS and/or antigen administration include but are not limited to topical, dermal, transdermal, transmucosal, epidermal parenteral, gastrointestinal, and nasopharyngeal and pulmonary, including transbronchial and transalveolar.

Preferred routes of dermal administration are those which are least invasive. Preferred among these means are transdermal transmission, epidermal administration and subcutaneous injection. Of these means, epidermal administration is preferred for the greater concentrations of APCs expected to be in intradermal tissue.

Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the ISS/antigen-containing composition to penetrate the skin and enter the blood stream. Compositions suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal

device (so-called "patch"). Examples of suitable creams, ointments etc. can be found, for instance, in the Physician's Desk Reference.

For transdermal transmission, iontophoresis is a suitable method. Iontophoretic transmission can be accomplished using commercially available patches which deliver their product continuously through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference. Other occlusive patch systems are also suitable.

For transdermal transmission, low-frequency ultrasonic delivery is also a suitable method. Mitragotri et al. (1995) *Science* 269:850-853. Application of low-frequency ultrasonic frequencies (about 1 MHz) allows the general controlled delivery of therapeutic compositions, including those of high molecular weight.

Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. Specifically, the irritation should be sufficient to attract APCs to the site of irritation.

An exemplary mechanical irritant means employs a multiplicity of very narrow diameter, short tines which can be used to irritate the skin and attract APCs to the site of irritation, to take up ISS-containing compositions transferred from the end of the tines. For example, the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France contains a device suitable for introduction of ISS-containing compositions.

The device (which is distributed in the U.S. by Connaught Laboratories, Inc. of Swiftwater, PA) consists of a plastic container having a syringe plunger at one end and a tine disk at the other. The tine disk supports a multiplicity of narrow diameter tines of a length which will just scratch the outermost layer of epidermal cells. Each of the tines in

the MONO-VACC kit is coated with old tuberculin; in the present invention, each needle is coated with a pharmaceutical composition of ISS/antigen-containing composition. Use of the device is preferably according to the manufacturer's written instructions included with the device product. Similar devices which can also be used in this embodiment are those which are currently used to perform allergy tests.

Another suitable approach to epidermal administration of ISS is by use of a chemical which irritates the outermost cells of the epidermis, thus provoking a sufficient immune response to attract APCs to the area. An example is a keratinolytic agent, such as the salicylic acid used in the commercially available topical depilatory creme sold by Noxema Corporation under the trademark NAIR. This approach can also be used to achieve epithelial administration in the mucosa. The chemical irritant can also be applied in conjunction with the mechanical irritant (as, for example, would occur if the MONO-VACC type tine were also coated with the chemical irritant). The ISS can be suspended in a carrier which also contains the chemical irritant or coadministered therewith.

Another delivery method for administering ISS-containing compositions makes use of non-lipid polymers, such as a synthetic polycationic amino polymer. Leff (1997) *Bioworld* 86:1-2.

Parenteral routes of administration include but are not limited to electrical (iontophoresis) or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Compositions suitable for parenteral administration include, but are not limited, to pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for injection of the ISS-containing compositions.

Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. The invention includes ISS-containing compositions suitable for gastrointestinal administration including, but not limited to, pharmaceutically acceptable, powders, pills or liquids for ingestion and suppositories for rectal administration.

Naso-pharyngeal and pulmonary routes of administration include, but are not limited to, inhalation, transbronchial and transalveolar routes. The invention includes ISS-containing compositions suitable for administration by inhalation including, but not limited to, various types of aerosols for inhalation, as well as powder forms for delivery systems.

Devices suitable for administration by inhalation of ISS-containing compositions include, but are not limited to, atomizers and vaporizers. Atomizers and vaporizers filled with the powders are among a variety of devices suitable for use in inhalation delivery of powders.

5 The methods of producing suitable devices for injection, topical application, atomizers and vaporizers are known in the art and will not be described in detail.

The choice of delivery routes can be used to modulate the immune response elicited. For example, IgG titers and CTL activities were identical when an influenza virus vector was administered via intramuscular or epidermal (gene gun) routes; however, the muscular inoculation yielded primarily IgG2a, while the epidermal route yielded mostly  
10 IgG1. Pertmer et al. (1996) *J. Virol.* 70:6119-6125. Thus, one skilled in the art can take advantage of slight differences in immunogenicity elicited by different routes of administering the immunomodulatory oligonucleotides of the present invention.

The above-mentioned compositions and methods of administration are meant to describe but not limit the methods of administering the antigen and/or ISS-containing  
15 compositions of the invention. The methods of producing the various compositions and devices are within the ability of one skilled in the art and are not described in detail here.

*Assessing modulation of the immune response*

Analysis (both qualitative and quantitative) of the immune response to ISS/antigen-containing compositions and to the second antigen or second antigen containing  
20 compositions can be by any method known in the art, including, but not limited to, measuring antigen-specific antibody production, activation of specific populations of lymphocytes such as CD4<sup>+</sup> T cells or NK cells, and/or production of cytokines such as IFN, IL-2, IL-4, or IL-12. Methods for measuring specific antibody responses include enzyme-linked immunosorbent assay (ELISA) and are well known in the art. Measurement of  
25 numbers of specific types of lymphocytes such as CD4<sup>+</sup> T cells can be achieved, for example, with fluorescence-activated cell sorting (FACS). Cytotoxicity assays can be performed for instance as described in Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523. Serum concentrations of cytokines can be measured, for example, by  
30 ELISA. These and other assays to evaluate the immune response to an immunogen are well known in the art. See, for example, *Selected Methods in Cellular Immunology* (1980) Mishell and Shiigi, eds., W.H. Freeman and Co.

Preferably, a Th1-type response is stimulated, i.e., elicited and/or enhanced. With reference to the invention, stimulating a Th1-type immune response can be determined *in vitro* or *ex vivo* by measuring cytokine production from cells treated with ISS as compared to those treated without ISS. Methods to determine the cytokine production of cells include those methods described herein and any known in the art. The type of cytokines produced in response to ISS treatment indicate a Th1-type or a Th2-type biased immune response by the cells. As used herein, the term "Th1-type biased" cytokine production refers to the measurable increased production of cytokines associated with a Th1-type immune response in the presence of a stimulator as compared to production of such cytokines in the absence of stimulation. Examples of such Th1-type biased cytokines include, but are not limited to, IL-2, IL-12, and IFN- $\gamma$ . In contrast, "Th2-type biased cytokines" refers to those associated with a Th2-type immune response, and include, but are not limited to, IL-4, IL-5, and IL-13. Cells useful for the determination of ISS activity include cells of the immune system, primary cells isolated from a host and/or cell lines, preferably APCs and lymphocytes, even more preferably macrophages and T cells.

Stimulating a Th1-type immune response can also be measured in a host treated with an ISS/antigen-containing composition and with a second antigen or second antigen containing composition and can be determined by any method known in the art including, but not limited to: (1) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower (or even absent) levels of IL-4 in an ISS-antigen treated and second antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS; (2) an increase in levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) in an ISS-antigen treated and second antigen treated host as compared to an antigen-primed or, primed and challenged, control treated without ISS; (3) IgG2a antibody production in an ISS-antigen treated and second antigen treated host as compared to a control treated without ISS; and/or (4) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even absent) levels of antigen-specific IgE in an ISS-antigen treated and second antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS. A variety of these determinations can be made by measuring cytokines made by APCs and/or lymphocytes, preferably macrophages and/or T cells, *in vitro* or *ex vivo* using

methods described herein or any known in the art. Methods to determine antibody production include any known in the art.

The Th1-type biased cytokine induction which occurs as a result of ISS administration produces enhanced cellular immune responses, such as those performed by NK cells, cytotoxic killer cells, Th1 helper and memory cells. These responses are particularly beneficial for use in protective or therapeutic vaccination against viruses, fungi, protozoan parasites, bacteria, allergic diseases and asthma, as well as tumors.

In some embodiments, a Th2 response is suppressed. Suppression of a Th2 response may be determined by, for example, reduction in levels of Th2-associated cytokines, such as IL-4 and IL-5, as well as IgE reduction.

### *Compositions of the invention*

The invention provides compositions comprising an immunomodulatory polynucleotide comprising an ISS and a carrier molecule. The compositions may further comprise an antigen (other than the carrier molecule) and/or a pharmaceutically acceptable excipient. Examples of carriers are provided above.

The invention also provides compositions comprising an immunomodulatory polynucleotide comprising an ISS-containing polynucleotide proximately associated with a first antigen, and further comprising a second antigen. First and second antigens have been described. As an example, in one embodiment, the first antigen is a conserved polypeptide and the second antigen is a variable polypeptide. One or more additional antigens may also be included in these compositions. In one embodiment, the first antigen is a viral conserved polypeptide, such as influenza NP (or an antigenic fragment of NP), and the second antigen is a viral coat polypeptide, such as influenza coat polypeptide (or an antigenic fragment thereof). These compositions, which generally contain an amount sufficient to elicit an antigen-specific immune response upon exposure to second antigen, may also comprise a pharmaceutically acceptable excipient and/or adjuvant.

The following examples are provided to illustrate, but not limit, the invention.



## EXAMPLES

### **Example 1. Effects of ISS + antigen co-administration on immune responses to unrelated antigens**

5 Experiments were performed in BALB/c mice to examine the effects of immunization of an Amb a 1-ISS conjugate (denoted "AIC"; the ISS-containing polynucleotide is 5'-TGA CTGTGAACGTTTCGAGATGA-3' (SEQ ID NO:1)) on immune responses to unrelated antigens or to non-specific mitogens. The first experiment demonstrated that co-administration of AIC shifts immune responses to the unrelated antigen  $\beta$ -galactosidase ( $\beta$ gal) toward a Th1-type response. The second experiment demonstrated that these effects of AIC on immune responses to  $\beta$ gal were diminished or absent if  $\beta$ gal was delivered four or eight weeks after AIC. The third experiment demonstrated that AIC delivered at the same time but at a different location than the delivery of  $\beta$ gal did not result in a Th-1 type response to the  $\beta$ gal.

#### *Co-administration of AIC shifts immune responses to $\beta$ gal toward Th1*

Mice were immunized intradermally (ID) three times at two-week intervals with either 1  $\mu$ g  $\beta$ gal, 1  $\mu$ g  $\beta$ gal mixed with 1  $\mu$ g AIC, or 1  $\mu$ g  $\beta$ gal mixed with 10  $\mu$ g AIC. Two weeks after the second and third immunizations,  $\beta$ gal-specific IgG1 and IgG2a responses were determined by ELISA as described in Raz et al. (1996) and Sato et al. (1996), and in the following Example.

The results of such an experiment are shown in Figure 1 and Table 2 (single asterisk indicates  $p < 0.05$  compared to  $\beta$ -gal alone; double asterisk indicates  $p < 0.005$  compared to  $\beta$ -gal alone; data shown as mean  $\pm$  standard deviation). After both the second and third immunizations, the antibody response to  $\beta$ gal alone was predominantly an IgG1 response, consistent with a Th2-type response. Co-administration of one or ten  $\mu$ g AIC with  $\beta$ gal significantly increased the IgG2a response to  $\beta$ gal after both the second and third immunization when compared to the IgG2a response seen with  $\beta$ gal alone, consistent with a Th1-type response. Co-administration of 1  $\mu$ g or 10  $\mu$ g AIC with  $\beta$ gal also reduced the anti- $\beta$ gal IgG1 responses when compared to the response with  $\beta$ gal alone.

**Table 2. Effects of AIC co-administration on antibody responses to  $\beta$ gal**

Group (dose)	Post 2nd Immunization Anti- $\beta$ gal Titers		Post 3rd Immunization Anti- $\beta$ gal Titers	
	IgG1	IgG2a	IgG1	IgG2a
$\beta$ gal (1 $\mu$ g) +AIC (1 $\mu$ g)	2401** $\pm$ 1936	5228* $\pm$ 7497	24600** $\pm$ 19840	30221* $\pm$ 41638
$\beta$ gal (1 $\mu$ g) +AIC (10 $\mu$ g)	7450* $\pm$ 7834	7065** $\pm$ 8464	59058 $\pm$ 33534	49515** $\pm$ 21507
$\beta$ gal (1 $\mu$ g) alone	18875 $\pm$ 15276	776 $\pm$ 1241	88405 $\pm$ 34327	7840 $\pm$ 8652

Four weeks after the third immunization, mice were sacrificed, and spleen cell IFN $\gamma$  and IL-5 responses to  $\beta$ gal were determined by ELISA. Generally, to measure cytokine secretion, splenocytes are harvested and resuspended in RP10 medium at  $5 \times 10^5$  splenocytes per well in 96 well flat-bottomed tissue culture microtiter plates. Culture medium alone as control or  $\beta$ gal is added to triplicate wells. Culture supernatants are sampled at 48 and 72 hours and then analyzed by ELISA for cytokine levels.

The results are shown in Table 3 (single asterisk indicates  $p < 0.05$  compared to  $\beta$ gal alone; double asterisk indicates  $p < 0.005$  compared to  $\beta$ gal alone; values are provided as mean  $\pm$  standard deviation, 5 spleen pools per group). Spleen cells from mice immunized with  $\beta$ gal alone secreted very little IFN $\gamma$  and relatively high levels of IL-5 in response to  $\beta$ gal. These responses indicate a Th2-type response and are consistent with the antibody responses discussed above. Co-administration of  $\beta$ gal with 1 or 10  $\mu$ g of AIC significantly increased the  $\beta$ gal specific IFN $\gamma$  responses and decreased the  $\beta$ gal-specific IL-5 responses by the spleen cells, again demonstrating a shift of the immune response to  $\beta$ gal in a Th1 direction.

**Table 3. IFN $\gamma$  and IL-5 secreted by spleen cells in response to  $\beta$ gal**

Group	IFN $\gamma$ (pg/ml)		IL-5 (pg/ml)	
	$\beta$ gal 25 $\mu$ g/ml	Media Alone	$\beta$ gal 25 $\mu$ g/ml	Media Alone
$\beta$ gal (1 $\mu$ g) +AIC (1 $\mu$ g)	469* $\pm$ 269	72 $\pm$ 19	136** $\pm$ 134	<32 $\pm$ —
$\beta$ gal (1 $\mu$ g) +AIC (10 $\mu$ g)	778** $\pm$ 300	77 $\pm$ 33	<32** $\pm$ —	<32 $\pm$ —
$\beta$ gal (1 $\mu$ g) alone	191 $\pm$ 119	<62 $\pm$ —	606 $\pm$ 338	<32 $\pm$ —

The results from this experiment demonstrate that co-administration of AIC with an unrelated antigen that normally gives a Th2-type response can significantly modulate the response to that antigen in a Th1-type direction.

The effects of AIC co-administration with  $\beta$ gal on anti- $\beta$ gal immune responses were statistically significant. For example, the IFN $\gamma$  responses to  $\beta$ gal were increased four-fold by co-administration of AIC with  $\beta$ gal. By comparison, administration of AIC increased IFN $\gamma$  responses by 26 to 90-fold compared to administration of Amb a 1 alone.

*AIC delivered four and eight weeks before  $\beta$ gal has little effect on immune responses to  $\beta$ gal*

In this experiment, mice (ten per group) were first immunized ID, twice at a two week intervals with 10  $\mu$ g AIC. Two separate lots of AIC induced similar anti-Amb a 1 IgG1 and IgG2a titers (see Table 4; titers < 120 were given a value of 120 for calculations; single asterisk indicates  $p < 0.05$  compared to the AIC naïve group; data shown as mean  $\pm$  standard deviation). Four or eight weeks after the second AIC dose, an immunization regimen with  $\beta$ gal was begun. Three 1  $\mu$ g doses of  $\beta$ gal were administered ID every two weeks at the same injection site as the AIC injection site. Mice originally receiving AIC lot "A" were immunized with  $\beta$ gal starting four weeks after the second AIC immunization and mice originally receiving AIC lot "B" were immunized with  $\beta$ gal starting eight weeks after the second AIC immunization. Each  $\beta$ gal immunization regimen included a control group

of naïve mice that had not previously been exposed to AIC or  $\beta$ gal. Two weeks after each  $\beta$ gal immunization, mice were bled and  $\beta$ gal-specific IgG1 and IgG2a responses were determined. The results are shown in Table 4. The data demonstrate that exposure to AIC four or eight weeks before immunization with  $\beta$ gal had no significant effect on the IgG1 or IgG2a responses to  $\beta$ gal when delivered at the same site. Immunization with AIC lot "B" did show a significant increase in IgG1 response to subsequent  $\beta$ gal immunization, but this increase was less than two-fold and was seen after the eight week rest but not after the four week rest.

**Table 4. Effect of previous immunization with AIC on antibody responses to  $\beta$ gal**

**Anti-Amb a 1 Titers**

Immunization Materials	Post 1st AIC		Post 2nd AIC	
	IgG1	IgG2a	IgG1	IgG2a
AIC 10 $\mu$ g $\beta$ gal 1 $\mu$ g 4 weeks post last AIC (lot "A")	303 $\pm 377$	6917 $\pm 2825$	90307 $\pm 55699$	351316 $\pm 161276$
$\beta$ gal 1 $\mu$ g No AIC	Not tested	Not tested	Not tested	Not tested

**Anti- $\beta$ -gal Titers**

Post 1st $\beta$ -gal		Post 2nd $\beta$ -gal		Post 3rd $\beta$ -gal	
IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
26413 $\pm 18645$	723 $\pm 717$	140106 $\pm 117783$	11708 $\pm 7286$	203103 $\pm 106489$	46156 $\pm 54214$
50745 $\pm 45982$	1152 $\pm 1957$	216887 $\pm 87938$	24552 $\pm 46992$	238867 $\pm 9201$	34323 $\pm 39687$

Immunization Materials	Post 1st AIC		Post 2nd AIC	
	IgG1	IgG2a	IgG1	IgG2a
AIC 10 $\mu$ g $\beta$ gal 1 $\mu$ g 8 weeks post last AIC (lot "B")	344 $\pm 212$	5462 $\pm 2985$	77840 $\pm 45790$	325703 $\pm 189856$
$\beta$ gal 1 $\mu$ g No AIC	Not tested	Not tested	Not tested	Not tested

Post 1st $\beta$ -gal		Post 2nd $\beta$ -gal		Post 3rd $\beta$ -gal	
IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
28612 $\pm 29004$	588 $\pm 695$	113689 $\pm 70880$	2264 $\pm 2713$	310363* $\pm 123199$	12918 $\pm 13112$
40242 $\pm 16079$	559 $\pm 1004$	141627 $\pm 85399$	1457 $\pm 1628$	186913 $\pm 94555$	3715 $\pm 2547$

Four weeks after the third  $\beta$ gal immunization, mice were sacrificed, spleens were harvested, and spleen cell cultures were stimulated for 4 days *in vitro* with  $\beta$ gal, as described above. IFN $\gamma$  and IL-5 levels in culture media were measured by ELISA. The results are shown in Table 5 (data shown as mean  $\pm$  standard deviation). Again, exposure to AIC four or eight weeks before  $\beta$ gal had no significant effect on the cytokine response of spleen cells to  $\beta$ gal.

**Table 5. Effect of previous immunization with AIC on spleen cell IFN $\gamma$  and IL-5 responses to  $\beta$ gal**

Immunization Group	IFN $\gamma$ (pg/ml) <sup>a</sup>			IL-5 (pg/ml)		
	Stimulated with			Stimulated with		
	$\beta$ -gal 25 $\mu$ g/ml	Amb a 1 25 $\mu$ g/ml	Media Alone	$\beta$ -gal 25 $\mu$ g/ml	Amb a 1 25 $\mu$ g/ml	Media Alone
A/C 10 $\mu$ g $\beta$ gal 1 $\mu$ g 4 weeks post last AIC	1433 $\pm$ 920	8255 $\pm$ 6146	332 $\pm$ 161	1095 $\pm$ 870	69 $\pm$ 69	<32 $\pm$ —
$\beta$ gal 1 $\mu$ g AIC Naïve	1080 $\pm$ 672	Not tested	945 $\pm$ 1117	1342 $\pm$ 926	Not tested	96 $\pm$ 180
AIC 10 $\mu$ g $\beta$ gal 1 $\mu$ g 8 weeks post last AIC	2582 $\pm$ 1137	11535 $\pm$ 5040	595 $\pm$ 429	2479 $\pm$ 1298	184 $\pm$ 107	<32 $\pm$ —
$\beta$ gal 1 $\mu$ g AIC Naïve	1799 $\pm$ 992	Not tested	957 $\pm$ 680	2367 $\pm$ 1880	Not tested	56 $\pm$ 63

This experiment demonstrates that while co-administration of AIC with  $\beta$ gal did have a significant effect on the quality of the immune response to  $\beta$ gal, this effect was not observed four or eight weeks after administration of AIC. AIC immunization four or eight weeks earlier had no significant effect on the immune response to subsequent  $\beta$ gal immunization as measured by antibody or cytokine responses.

*AIC delivered at the same time but at a different site has little effect on the immune response to  $\beta$ gal*

In this experiment, the anti- $\beta$ gal IgG1 and IgG2a responses were compared in groups of ten mice immunized with  $\beta$ gal alone (intramuscularly (IM) in the thigh or ID in the tail),  $\beta$ gal co-administered with 1 or 10  $\mu$ g of AIC at the same site (IM, thigh or ID, tail), or  $\beta$ gal delivered at one site (IM, thigh) and 1 or 10  $\mu$ g of AIC delivered at a distant site (ID, tail). One  $\mu$ g doses of  $\beta$ gal were used throughout this experiment. All animals received two injections of the immunogens at a two week interval. Two weeks after each injection, animals were bled and anti- $\beta$ gal IgG1 and IgG2a titers were determined by

ELISA. The results are shown in Table 6 (data shown as mean  $\pm$  standard deviation; single asterisk indicates  $p < 0.05$  compared to Amb a 1 by the appropriate route; double asterisk indicates  $p < 0.005$  compared to Amb a 1 by the appropriate route).

Table 6. Effects of AIC delivered at a separate site on immune responses to  $\beta$ gal

Immunization Materials	Anti- $\beta$ -gal Titers			
	Post 1st		Post 2nd	
	IgG1	IgG2a	IgG1	IgG2a
AIC 1 $\mu$ g ID: $\beta$ gal 1 $\mu$ g IM	4060 $\pm 4717$	353 $\pm 514$	44538 $\pm 54492$	3723 $\pm 6171$
AIC 10 $\mu$ g ID: $\beta$ gal 1 $\mu$ g IM	1747 $\pm 2549$	429 $\pm 872$	86630 $\pm 78519$	10438* $\pm 17436$
AIC 1 $\mu$ g + $\beta$ gal 1 $\mu$ g co-administered IM	2740 $\pm 1215$	3711** $\pm 2895$	51055 $\pm 34682$	20909** $\pm 17983$
AIC 10 $\mu$ g + $\beta$ gal 1 $\mu$ g co-administered IM	3144 $\pm 3201$	24106* $\pm 13343$	84608 $\pm 68179$	139930** $\pm 79117$
$\beta$ gal 1 $\mu$ g IM	5959 $\pm 5686$	151 $\pm 182$	60149 $\pm 63204$	2575 $\pm 4521$

Immunization of mice with  $\beta$ gal alone by IM injection resulted in relatively high IgG1 responses and relatively low IgG2a responses, consistent with experiments described above and representative of a Th2-type immune response. When  $\beta$ gal was co-administered with AIC (1 or 10  $\mu$ g) IM, large increases in the anti- $\beta$ gal IgG2a response was seen, consistent with the experiment described above. After the first immunization, co-administration of 1  $\mu$ g AIC increased anti- $\beta$ gal IgG2a responses by 25-fold compared to  $\beta$ gal alone. Co-administration of 10  $\mu$ g AIC increased IgG2a responses 159-fold. After the second immunization, co-administration of 1  $\mu$ g AIC with 1  $\mu$ g  $\beta$ gal increased anti- $\beta$ gal IgG2a responses by 8-fold. Co-administration of 10  $\mu$ g AIC with  $\beta$ gal increased IgG2a responses 54-fold. These differences are highly statistically significant ( $p < 0.005$ ).

In contrast to co-administration at the same site, delivery of AIC at a distant site had little effect on anti- $\beta$ gal IgG2a responses. Same day injection of 1  $\mu$ g AIC ID in the tail and 1  $\mu$ g  $\beta$ gal IM in the thigh increased anti- $\beta$ gal IgG2a responses only 2.3 fold after the

first immunization and 1.4 fold after the second, compared to administration of  $\beta$ gal alone. Similarly, using 10  $\mu$ g AIC at the distant site increased anti- $\beta$ gal responses only 2.8-fold after the first immunization and only 4-fold (although statistically significant,  $p < 0.05$ ) after the second compared to  $\beta$ gal alone.

5 AIC at either dose had little effect (three-fold or less) on anti- $\beta$ gal IgG1 responses.

In this experiment, co-delivery of AIC and an unrelated antigen to the same site dramatically increased the Th1 immune response to the unrelated antigen, while delivery of AIC to a site distant from the unrelated antigen had little effect on the Th1 response to that antigen.

10

#### **Example 2. Administration of ISS with Influenza Nucleoprotein (NP)**

##### *Conjugation of oligonucleotide with influenza NP protein and NP peptide*

Influenza NP is prepared from purified influenza virus (such as A/Taiwan/86, H1N1) as described in, for example, Albo et al. (1995) *J. Virol.* 69:3799-3806 and Cooper et al. (1996) *J. Inf. Diseases* 173:279-284). Purified influenza virus (such as A/Taiwan/86, H1N1) is purchased from Research Diagnostics (Flanders, NJ).

A synthetic peptide for use in ISS conjugates represents amino acids 206-229 of influenza NP and is prepared by standard solid-phase chemistry. This peptide contains a strong, conserved T helper cell (Th) epitope that is recognized by BALB/c mice (Gao et al. (1989) *J. Immunol.* 143:3007-3014). This epitope is the most commonly recognized NP epitope in human subjects, with 48% of individuals showing helper T cell recognition (Brett et al. (1991) *J. Immunol.* 147:984-991). This epitope has demonstrated only one amino acid change (at positions 218) in influenza isolates between 1934 and 1975, and no amino acid changes from 1975 through 1990 across H1N1, H2N2 and H3N2 strains (Shu et al. (1993) *J. Virol.* 67:2723-2729).

Synthetic peptides such as NP 206-229 (FWRGENGRKTRSAYERMCNILKGK (SEQ ID NO:9)), NP 147-155 (TYQRTRALV (SEQ ID NO:10)), HA 111-119 (FERFEIFPK (SEQ ID NO:11)) and HA 533-541 (IYSTVASSL (SEQ ID NO:12)) are purchased from Applied Biosystems (Foster City, CA). NP 206-219 represents a NP H-2<sup>d</sup> Th epitope described above. NP 147-155 represents a conserved NP H-2<sup>d</sup> CTL epitope that showed no variation in influenza isolates from 1934 through 1990 (Fu et al. (1997) *J. Virol.* 71:2715-2721). HA 111-119 represents an HA H-2<sup>d</sup> Th epitope conserved across all H1N1

strains (Hackett et al. (1983) *J. Exp. Med.* 158:294-302). HA 533-541 represents an HA H-2<sup>d</sup> CTL epitope that is found in H1N1 and H2N2 strains (Tamura et al. (1998) *J. Virol.* 72:9404-9406).

5' Thio phosphorothioate ISS oligonucleotides are conjugated to lysine  $\epsilon$ -amino groups of both NP and NP peptide using the heterobifunctional cross-linking agent sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate) by the following technique. Five milligrams of NP (100 nmole) or 5 milligrams of NP peptide (2  $\mu$ mole) is treated with 30-90 fold excess of NEM (N-ethylmaleimide) and 20-50 fold excess of sulfo-SMCC at room temperature for 2 hours. The maleimido-modified NP or NP peptide is purified from unreacted reagents by gel filtration chromatography on a G-25 desalting column. The ISS oligonucleotide used in the conjugate is 5'-TGACTGTGAACGTTTCGAGATGA-3' (SEQ ID NO:1) (Hybridon Speciality Products). A 5'-disulfide modified ISS oligonucleotide (Glen Research, 5'-disulfide Modifier 6) is reduced to the thiol by treatment with tris (2-carboxyethyl)phosphine (TCEP) in PBS. Following purification by gel filtration chromatography on a G-25 desalting column, 5-20 molar excess of the thiol-activated oligonucleotide is incubated with the maleimido-modified NP or NP peptide at room temperature for three hours. The NP-ISS conjugate or NP peptide-ISS conjugate is purified using gel filtration chromatography.

Identical conjugates are made to NP or NP peptide with a control, non-ISS oligonucleotide.

Successful conjugation of NP or NP peptide to ISS is confirmed by non-reducing SDS-PAGE combined with coomassie blue and oligonucleotide-specific silver staining. Quantitation of the oligonucleotide/protein molar ratio is determined by the ratio of the molar oligonucleotide content divided by the molar protein content. The oligonucleotide content is determined by A260 nm absorbance readings and the protein content is determined by BCA assays.

#### *Immunization and Immune Response*

Generally, for these experiments, groups of ten, six to eight week old female BALB/c mice are immunized twice (two week interval) intradermally with 1  $\mu$ g of either ISS-NP conjugate, ISS mixed with NP in 50  $\mu$ l saline, ISS-NP peptide conjugate, NP-control oligo conjugate, NP peptide-control oligo conjugate, NP alone in 50  $\mu$ l saline, NP



peptide alone or PBS. Mice are bled every two weeks post-immunization, serum separated and stored at -20°C for later analysis. At week 6, spleens are harvested and splenocytes prepared. Naïve mice are also included in the experiments. The cytokine secretion profile, antigen-dependent antibody responses and antigen-dependent CTL responses of these mice are tested *in vitro*.

#### Antibody Assays

Mice are bled via the sinus orbital at 2 weeks post each immunization. Serum is prepared and assayed for anti-NP or anti-HA isotype response by ELISA. Briefly, Nunc Maxisorp plates are coated with either NP or vaccine material (as HA source) overnight at 4°C. Plates are washed and blocked, then samples loaded, serially diluted and incubated overnight at 4°C. Duplicate samples are assayed on separate plates. Plates are again washed and biotinylated goat anti-mouse isotype-specific detection antibody is loaded. After 1 hour at room temperature, plates are again washed and streptavidin-horseradish peroxidase (HRP) is loaded into wells. Plates are incubated again for 1 hour at room temperature and then washed. Plates are developed with tetramethyl benzidine (TMB) for 10 minutes at room temperature and color development stopped with 2M H<sub>2</sub>SO<sub>4</sub>. Optical densities are read at 450 nm with background subtraction done at 650 nm. Antibody titers are reported as the reciprocal of the highest dilution giving an OD<sub>450</sub>=0.5.

#### Cytokine Assays

At 4 weeks post second immunization, spleens are harvested and assayed individually for IFN $\gamma$  and IL-5 production following *in vitro* stimulation with either NP protein, NP peptides (such as NP 206-229) or HA peptides (such as HA 111-119 and HA peptides from H1 strains). Briefly, spleens are dissociated through a wire screen, washed and cells counted.  $2 \times 10^5$  cells/well are plated into 96-well flat bottom tissue culture plates and NP or HA peptide is added to the media. Plates are incubated for 4 days and supernatants harvested and stored at -80°C until assayed for cytokines by ELISA. Briefly, Nunc Maxisorp plates are coated with either anti-IFN $\gamma$  or anti-IL5 monoclonal antibody overnight at 4°C. Plates are washed and blocked, then standards and samples loaded, serially diluted and incubated overnight at 4°C. Duplicate samples are assayed on separate plates. Plates are again washed and biotinylated anti-IFN $\gamma$  or anti-IL5 (MAbs) are loaded. After 1 hour at room temperature, plates are again washed and streptavidin-HRP is loaded into wells. Plates are incubated again for 1 hour at room temperature and then

washed. Plates are developed with TMB for 10 minutes at room temperature and color development stopped with 2M H<sub>2</sub>SO<sub>4</sub>. Optical densities are read at 450 nm with background subtraction done at 650 nm. Cytokine concentration of unknown supernatants are read from the standard curve.

5        CTL Assays

At 4 weeks post second immunization, spleens are harvested and assayed for cytotoxic activity following *in vitro* stimulation with peptides specific for CTL epitopes of either NP or HA, such as NP 147-155 or HA 533-541. Briefly, spleens are dissociated through a wire screen, washed and cells counted. 5 x 10<sup>6</sup> cells are stimulated with peptide (1 µg/ml) for 1 hour at 37°C and then washed. Peptide stimulated cells (1 x 10<sup>6</sup> cells/well) and non-stimulated spleen cells (4 x 10<sup>6</sup> cells/well) are plated together into 24-well flat bottom tissue culture plates in media containing Rat T-Stim. Plates are incubated at 37°C with 7% CO<sub>2</sub> for 7 days. Cells are fed on Day 3 and washed and replated on Day 5. On Day 7, target cells (SV Balb) are peptide pulsed, <sup>51</sup>Cr loaded for 1 hour at 37°C and then washed. The Day 7 effector cells are counted and plated to achieve various effector:target ratios (60:1, 12:1, 2.4:1). Target cells are plated (5000 cells/well) and incubated at 37°C for 4 hours. Supernatant from each well is then counted for <sup>51</sup>Cr release and % lysis is calculated.

15        Influenza infection

20        Infectious influenza virus (mouse adapted), such as A/Taiwan/1/86 (H1N1), is delivered intranasally, dropwise using a micropipettor. For a lethal challenge, the virus is titered to establish an LD<sub>50</sub> and is delivered in a 50 µl volume. For a sublethal challenge, the virus is titered to a dose that is non-lethal but results in infection of the mouse. After the dose is delivered, the mouse is monitored for the next two weeks to confirm infection. Symptoms of infection include weight loss, decrease in activity and/or scruffy fur.

25        Enhancement of Th1 responses to other influenza vaccine antigens by co-injection of NP-ISS compositions with influenza vaccine

Groups of fifteen, six to eight week old female BALB/c mice are immunized twice (at a two week interval) intradermally with either NP-ISS conjugate (1 µg) + whole-inactivated influenza vaccine, such as made from A/Taiwan/86 virus, at a dose containing 1 µg HA, NP peptide-ISS conjugate (1 µg) + influenza vaccine, NP (1 µg) + influenza vaccine, NP peptide (1 µg) + influenza vaccine, influenza vaccine mixed with a molar

equivalent of ISS oligonucleotide or influenza vaccine alone. Mice are bled every two weeks post-immunization, serum separated and prepared for analysis. At week 6, spleens are harvested from five animals per group and splenocytes prepared. Splenocytes are stimulated *in vitro* with NP or an HA peptide, such as an HA peptide specific for a Th epitope of H1 strains (such as HA 111-119), then culture supernatants are assayed for IFN $\gamma$  and IL-5. At the same time point, splenocytes are stimulated with an NP peptide or HA peptide, such as NP 147-155 or an HA peptide that is cross-reactive to H1 and H2 CTL epitopes (such as HA 533-541), then assayed for cytotoxic activity. The remaining animals are challenged intranasally with 5 LD<sub>50</sub> of influenza virus, such as A/Taiwan/86, and monitored for 14 days for mortality and morbidity (such as weight loss, decrease in activity and/or scruffy coat).

Effect of pre-injection of NP-ISS on Th1 responses to NP and other viral proteins upon subsequent influenza virus infection.

Mice receive intradermal immunizations with 1  $\mu$ g doses of NP-ISS conjugate, NP + ISS mixture, NP peptide-ISS conjugate, NP-control oligo conjugate, NP alone, NP peptide alone or PBS as described above. Mice are bled every two weeks post-immunization, serum separated and stored at -20°C for later analysis. At week 6, mice are challenged with a sub-lethal dose of influenza virus, such as A/Taiwan/86, via the nasal route. The rate and severity of infection is monitored as compared to mice not treated with NP/ISS compositions. Mice are bled at week 8 and serum prepared. Antibody isotype responses to NP and HA are assayed. At week 10, spleens are harvested and splenocytes are stimulated *in vitro* with NP or HA peptides, such as NP 206-229 peptide or HA 111-119 peptide, then culture supernatants are assayed for IFN $\gamma$  and IL-5 levels. At the same timepoint, splenocytes are stimulated with HA or NP peptides, such as the CTL peptides HA 533-541 or NP 147-155, then assayed for cytotoxic activity.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the descriptions and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

What is claimed is:

1. A method of modulating an immune response to a second antigen in an  
5 individual, comprising administering to the individual an immunomodulatory  
polynucleotide comprising an immunostimulatory sequence (ISS) and a first antigen,  
wherein the polynucleotide and first antigen are administered in an amount sufficient to  
modulate an immune response to the second antigen upon exposure to the second antigen.
- 10 2. The method of claim 1, wherein the first antigen administered in the absence of  
the second antigen, and exposure to the second antigen occurs concurrently with exposure  
to the first antigen.
3. The method of claim 1, wherein the immunomodulatory polynucleotide and first  
15 antigen are proximately associated.
4. The method of claim 3, wherein the immunomodulatory polynucleotide and first  
antigen are conjugated.
- 20 5. The method of claim 3, wherein the immunomodulatory polynucleotide and first  
antigen are proximately associated by a platform molecule.
6. The method of claim 3, wherein the immunomodulatory polynucleotide and first  
antigen are proximately associated by encapsulation.
- 25 7. The method of claim 1, wherein the immunomodulatory polynucleotide and first  
antigen are administered in an admixture.
8. The method of claim 1, wherein the ISS and first antigen are administered  
30 before the individual is exposed to the second antigen.

9. The method of claim 8, wherein the ISS and first antigen are administered less than about 10 days before exposure to the second antigen.

5 10. The method of claim 1, wherein the ISS and first antigen are administered upon exposure to second antigen.

10 11. The method of claim 1, wherein the immunomodulatory polynucleotide and first antigen are administered at a site in the individual which is the same as the site of exposure to the second antigen.

12. The method of claim 1, wherein the immunomodulatory polynucleotide and first antigen are administered at a site in the individual which is different from the site of exposure to the second antigen.

15 13. The method of claim 1, wherein the first antigen is an allergen.

14. The method of claim 1, wherein the first antigen is a conserved polypeptide of a virus.

20 15. The method of claim 14, wherein the conserved viral polypeptide is influenza nucleocapsid protein.

25 16. The method of claim 14, wherein the conserved viral polypeptide is human immunodeficiency virus (HIV) gag protein.

17. The method of claim 1, wherein the first antigen is a carrier molecule.

30 18. The method of claim 17, wherein the carrier molecule is diphtheria toxin mutant (CRM 197).

19. The method of claim 17, wherein the carrier molecule is diphtheria toxoid.

20. The method of claim 1, wherein the first antigen is associated with a carrier molecule.

5 21. The method of claim 1, wherein the immune response is modulated by stimulating a Th1 response to the second antigen.

22. The method of claim 21, wherein production of Th1-associated antibodies is stimulated.

10 23. The method of 21, wherein interferon gamma production is stimulated.

24. The method of claim 1, wherein the ISS comprises the sequence 5'-cytosine, guanine-3'.

15 25. The method of claim 24, wherein the ISS comprises the sequence 5'-TCG-3'.

26. The method of claim 24, wherein the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3'.

20 27. The method of claim 26, wherein the ISS comprises the sequence 5'-AACGTT-3'.

25 28. The method of claim 26, wherein the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'.

29. The method of claim 26, wherein the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

30 30. The method of claim 26, wherein the ISS comprises a sequence selected from the group consisting of AACGTTCC, AACGTTCCG, GACGTTCC, and GACGTTCCG.

31. The method of claim 29, wherein the ISS comprises the sequence  
TGA CTGTGAACGTTTCGAGATGA (SEQ ID NO:1).

32. The method of claim 1, wherein the individual is a mammal.

5

33. The method of claim 32, wherein the mammal is human.

34. A composition comprising an immunomodulatory polynucleotide comprising  
an ISS and a carrier molecule.

10

35. A composition comprising an immunomodulatory polynucleotide comprising  
an ISS proximately associated with a first antigen and further comprising a second antigen.

36. The composition of claim 35, wherein the immunomodulatory polynucleotide  
is proximately associated to first antigen by conjugation.

15

37. The composition of claim 35, wherein the first antigen is a viral conserved  
polypeptide and the second antigen is a viral variable polypeptide.

# Effect of AIC Co-administration on Anti-Bgal IgG2a responses

1/1  
Figure 1

